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THE JOURNAL OF HYGIENE

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CONTENTS

No. 1 (OCTOBER 1923)

	PAGE
SMITH, J. A Study of Diphtheria Bacilli, with Special Reference to their Serological Classification	1
RHODES, E. C. Notes on the Spread of Bacterial Infection. (With 2 Charts)	6
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part II. (With Charts III–XII)	12
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part III. (With Charts XIII–XVI)	37
KENT, S. S. SHRI. The Nutrition of Bacteria, with Special Reference to <i>Bacillus influenzae</i> (Pfeiffer). (With Plate I)	52
PECKHAM, C. F. An Outbreak of Pork Pie Poisoning at Derby. (With a Foreword by William G. Savage)	69
SAWYER, W. A., SWEET, W. C. and SHAW, A. ELAND. Institutional Hookworm Disease in a Non-Endemic Region. (With 1 Text-figure)	77
ANDERSON, JAMES S., KINLOCH, J. PARLANE and SMITH, J. Epidemic Enteritis in Aberdeen due to Food Infections	89
LEARMONTH, J. R. On the Inheritance of Acquired Antibodies	100
EAGLETON, A. J. and BAXTER, EDITH M. The Serological Classification of <i>Bacillus diphtheriae</i>	107

No. 2 (DECEMBER 1923)

FORBES, J. GRAHAM. The Atmosphere of the Underground Electric Railways of London. A Study of its Bacterial Content in 1920. (With 1 Text-figure and 6 Charts)	123
SHOUSH, A. T. Spontaneous Agglutination of the Cholera Vibrio in Relation to Variability	156
SIMPSON, J. V. A. A Report on the Ventilation of Schools. (With 1 Graph)	164
MURRAY, E. G. D. Some Aspects of Meningococcal Virulence. A Report to the Medical Research Council of work carried out at the University of Cambridge Field Laboratories	175
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part IV. (With Charts XVII–XXI)	208

	PAGE
TOPLEY, W. W. C. and AYRTON, JOYCE. A Technique for Measuring the Excretion of Bacilli of the Enteric Group in the Faeces of Infected Mice. (With 1 Chart)	222
TOPLEY, W. W. C. and AYRTON, JOYCE. The Excretion of <i>B. enteritidis</i> (<i>aertrycke</i>) in the Faeces of Mice after Administration by Mouth. (With 10 Charts and 1 Text-figure)	234

No. 3 (MARCH 1924)

GREENWOOD, MAJOR. The Life and Scientific Work of Arthur William Bacot. With a chapter by J. A. ARKWRIGHT. (With Portrait, Plate II)	265
TOPLEY, W. W. C. and AYRTON, JOYCE. The Segregation of Biological Factors in <i>B. enteritidis</i> (<i>Aertrycke</i>). A Report to the Medical Research Council. (With Plate III)	305
DE SMIDT, F. P. G. Notes on the Sporulation of <i>B. sporogenes</i> and other Anaerobes. A Report of the Food Investigation Board. (With 1 Chart)	314
DE SMIDT, F. P. G. An Apparatus for anaerobic Plate Cultivation in Hydrogen for separate Petri Capsules. (With Plate IV)	325
TEH, WU LIEN- (TUCK, G. L.). A Further Note on Natural and Experimental Plague in Tarbagans	329
KHALED, Z. A Comparative Bacteriological Study of Bovine Abortion and Undulant Fever	335
BAMFORTH, J. A small Outbreak of Dysentery associated with an Unusual Bacillus	343
DUDGEON, LEONARD S. Acute Infection of the Urinary Tract due to a Special Group of Haemolytic Bacilli. (With 2 Charts)	348
EASTWOOD, ARTHUR. The Capillary Endothelium in Relation to Antibodies	355

No. 4 (JULY 1924)

COBBETT, LOUIS. Vegetable Decomposition in Ditch Water Simulating Sewage Contamination. (With Plate V)	389
PETRIE, G. F. A Commentary on Recent Plague Investigations in Transbaikalia and Southern Russia	397
DUNCAN, J. T. A "New" Salmonella from a Case of Enteric Fever. (With 4 figures)	402

Contents

vii

PAGE

SADLER, WILFRID, KELLY, C. D. and MARTIN, G. R. On the Producing of Milk having a Low Bacterial Content	410
MACCONKEY, A. T. On the Concentration of Serum by means of Sodium Sulphate	413
WATT, JAMES P. Typhoid Carriers in Aberdeenshire	417
CARLETON, H. M. The Pulmonary Lesions produced by the Inhalation of Dust in Guinea-Pigs. A Report to the Medical Research Council. (With Plates VI-X)	438
MACCONKEY, A. T. The Stability of Tetanus Toxin in 50 % Glycerine and of Tetanus Antitoxin in Saturated Salt Solution	473
INDEX OF AUTHORS	477
INDEX OF SUBJECTS	479

A STUDY OF DIPHTHERIA BACILLI, WITH SPECIAL REFERENCE TO THEIR SEROLOGICAL CLASSIFICATION.

By J. SMITH, M.D., D.P.H.

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ABSTRACT.

MUCH work has already been done in connection with the differentiation of true diphtheria bacilli from diphthomorphic organisms. Some observers have maintained that all disease producing strains could be distinguished from non-pathogenic types by their sugar reactions. Others have laid much stress on some particular forms of virulence test. More recently serological classification has been attempted in order to help to solve this problem.

With regard to sugar reactions Graham-Smith (1908) found that certain strains produced acid in media containing saccharose, while Hine (1913), on the other hand, maintained that no true diphtheria bacillus could ferment this sugar. Eagleton and Baxter (1922) have shown that diphtheria bacilli, virulent and non-virulent, ferment glucose but do not produce any change in saccharose. Jordan and his collaborators (1922) have found that virulent and non-virulent bacilli fermented glucose and maltose and produced no change in saccharose and that their action on dextrin was variable.

Work on the serological classification of these organisms has also shown some considerable variation. Havens (1920) isolated 206 strains of diphtheria bacilli. These represented cultures from acute cases, release cultures and cultures from healthy carriers. He found that these 206 strains could be divided into two serological groups, one group containing 169 strains, the other 37. There was no evidence of any cross agglutination. Durand (1920) isolated 255 strains of typical diphtheria bacilli and after excluding those strains which would not form stable suspensions divided them into five groups, A. B. C. D. and E. containing 16, 8, 25, 61 and 40 strains respectively. Eighty-seven strains were found to be inagglutinable by these five sera but nevertheless could absorb agglutinins from such sera. His final figures for the five groups of bacilli are 18, 8, 31, 76 and 51. Durand and Guerin (1921) in a further paper showed that small isolated outbreaks were always due to the same type of bacillus. They also found that healthy carriers gave the same type of bacillus as was found in the cases arising from these sources of infection. Bell (1922) isolated 133 strains of *B. diphtheriae* indifferently collected from cases and carriers. He was able to agglutinate 80 per cent. of these strains by three monovalent sera. Group I contained 17 strains, Group II 8 strains, Group III 80 strains.

In view of the results which have just been described it has appeared important to investigate further a considerable number of strains as encountered in routine work, not only as regards the serological characteristics of the organisms but also in regard to their morphology, their biochemical reactions, and their virulence for guinea-pigs.

TECHNIQUE.

Strains of diphtheria bacilli were collected from acute cases only. Strains which were isolated from cases without evidence of clinical diphtheria were discarded. Pure cultures having been obtained an 18-hour serum culture was used for studying the morphology and the morphological characteristics were noted according to the Wesbrook classification. Stock cultures were maintained on gelatin slants in the cool incubator at 20° C. The biochemical reaction of the various strains were tested in Hiss's serum water medium containing 1 per cent. of the various sugars—glucose, galactose, laevulose, maltose, dextrin and saccharose. Litmus was used as an indicator. The power of the organism to break down two alcohols, namely glycerol and mannitol, was also tested.

Many of the strains were tested for virulence by guinea-pig inoculation. In order to obtain a uniform amount of growth all strains were subcultured four times in a dextrose free broth containing 2 per cent. of Witt's peptone. The hydrogen ion concentration of this broth after sterilisation was pH 7.4. A fifth subculture was then made and incubated for 48 hours at 37° C. and 0.5 c.c. of the broth culture per 100 grams of weight of the guinea-pig was injected subcutaneously. A control animal was used on all occasions. This animal received the same amount of culture as was given to the test guinea-pig together with 500 units of antitoxin.

For the preparation of agglutinating sera half grown rabbits were used and the antigen was given intravenously in all cases. At first, the antigen employed was a formalised suspension of diphtheria bacilli which had been grown on horse serum slants. Some rabbits died from the toxic effects, but it was soon discovered that the response as measured by the agglutinins present in rabbits' serum was very poor and this method was abandoned. Living bacilli were then used as antigen. An 18-hour culture on a serum slant was washed off with 10 c.c. of salt solution and thoroughly emulsified. The requisite amount of suspension was incubated at 37° C. along with the necessary amount of antitoxin for one hour before being injected.

The following table serves to indicate in general the amounts of culture and antitoxin used:

Day	Amount of Serum slant	Antitoxin
1st	0.1	1000
6th	0.2	1000
11th	0.4	500
16th	0.6	500
21st	0.8	200

Sera with titres ranging between 1-1600 and 1-12,800 were obtained by this method.

The method of preparing the agglutination tests was the usual one of doubled dilution. The tests were set up in tubes placed in racks as devised by Dreyer and incubated for two hours at 37° C. A control tube containing salt solution and bacillary emulsion was used in connection with each test. All bacterial suspensions were standardised by the method of Kirwan and Brown (1915). Occasionally considerable difficulty was encountered owing to strains showing persistent spontaneous agglutination.

DISCUSSION OF RESULTS.

1. *The Biochemical Reaction.* The sugar reaction of 89 strains was tested by using Hiss's serum water medium as a basis. The reactions were noted after three days' incubation and again after ten days' incubation. Within the three-day incubation period all strains were found to produce acid in glucose, galactose, laevulose, maltose and dextrin. On glycerol the reaction varied, sometimes acid was produced and at other times no change was observed. On saccharose and mannite uniformly negative results were obtained. The production of clot in the media was usually well established within the three days' period on glucose, galactose, laevulose, and maltose. The action on dextrin and glycerol was variable. At the end of ten days' incubation very few strains had failed to produce acid and clot on glucose, galactose, laevulose, and maltose. On dextrin in which all strains had already produced acid, 34 strains failed to produce clot. On mannite and saccharose there was again no change noted.

2. *Virulence Test.* By the method already described 52 strains were tested for virulence as soon after isolation as possible. Forty-six strains were found to kill the guinea-pigs within four days. In nine instances the guinea-pigs died within 24 hours after being inoculated. Thirty strains killed in two days, six strains killed in three days, and only one guinea-pig died on the fourth day. All control animals survived.

The six non-virulent strains were isolated from ordinary cases of diphtheria. In two instances it was found that the strain isolated from the nose was non-virulent while the strain isolated from the throat was virulent.

3. *Serological Classification.* Eighty-nine strains were collected from eighty clinical cases of diphtheria, in nine of the cases the organism being isolated from both the throat and nose. It was found, however, as the investigation proceeded that the strains isolated from the throat and nose of the same patient belonged without exception to the same serological group. Accordingly 80 strains of diphtheria bacilli were grouped as follows: Type I, 68 strains; Type II, one strain; Type III, six strains; Type IV, one strain; Type V, two strains; Type VI, one strain; Type VII, one strain.

Type I serum had a titre of 1 in 1600. Strains belonging to other types were not agglutinated in a greater dilution than 1 in 100.

Type II serum with a titre of 1 in 1600 only agglutinated the homologous strain to titre. It agglutinated Type I strains to 1 in 100 dilution and the other types in dilution of 1 in 50 to 1 in 200 only.

Type III serum agglutinated seven strains to full titre (1 in 1600). It agglutinated Type I strains to a dilution of 1 in 400, but strains belonging to other types were only agglutinated up to 1 in 50 dilution or not at all.

Type IV serum had a titre of 1 in 6400 and only agglutinated the homologous strain to full titre. It agglutinated Type I strains to 1 in 100 dilution of the serum, but strains belonging to other types were unaffected in this dilution.

Type V serum had a titre of 1 in 6400 and again only agglutinated the homologous strain to titre. Type I strains were agglutinated to 1 in 800. Strains belonging to other types were not agglutinated above 1 in 100.

Type VI serum only agglutinated the homologous strain to titre, 1 in 1600. Strains of Types II and III were unaffected by a 1 in 100 dilution. Strains belonging to Types IV and V were agglutinated to 1 in 100. Strains belonging to Type I were agglutinated to 1 in 200.

Type VII serum, with a titre of 1 in 3200, only agglutinated one strain. No other strain was agglutinated to titre. Strains of Types II, III and IV were not agglutinated by 1 in 100 dilution. Type V strains were agglutinated by 1 in 100, Type I strains by 1 in 400 dilution.

4. *Serological Groups in Relation to Morphology.* It was thought at first that strains of diphtheria bacilli having the same morphological characteristics might also correspond in their serological reactions. All strains were classified according to the Westbrook classification, but no relationship between the morphological classification and the serological classification could be established.

5. *Serological Groups in Relation to Biochemical Reactions.* As the biochemical reactions were very similar for all strains no grouping of the strains on this basis was possible.

6. *Serological Groups in Relation to Virulence Tests.* Of 52 strains tested for virulence 38 belonged to Group I. All but six of these were found to be virulent. The remaining 14 strains included all strains belonging to the other six groups. These were all virulent. It is of interest to note that although one batch of antitoxin was used throughout in the protection of control animals none of these control animals died.

7. *Serological Groups in Relation to Cases.* It has already been noted that strains isolated from the nose and throat of the same patient always belong to the same serological group. It has further been found that when two or more cases of diphtheria occurred in the same family the bacilli isolated from these cases also belonged to the same serological group.

The following table shows the mortality rates for the various groups:

						No. of cases	No. of deaths	No. of cases in which paralysis occurred
Cases from which Type I			strains were isolated			68	4	2
"	"	"	II	"	"	1	0	0
"	"	"	III	"	"	6	1	0
"	"	"	IV	"	"	1	0	1
"	"	"	V	"	"	2	2	0
"	"	"	VI	"	"	1	0	0
"	"	"	VII	"	"	1	1	0

As the number of cases harbouring bacilli other than Type I is small no conclusions can be drawn as to one type being more virulent than another.

It has already been demonstrated by Havens (1920) who classified his strains into two groups that when one minimal lethal dose of toxin of a Group I strain together with five units of standard antitoxin (made from the toxin of a Group I bacillus) was injected into a guinea-pig, the animal survived. When the same dose of toxin of a Group II strain was given with the antitoxin the guinea-pig died. In the case of Group II toxin 20 units of the standard antitoxin were required to protect against 1 unit of toxin. These findings of Havens have not been confirmed. On the contrary, Hartley (1922) has found that the antitoxin made from the toxin of one serological type afforded equal protection against the toxins of other types.

SUMMARY.

1. The fermentation of glucose and non-fermentation of saccharose was a constant finding with 80 strains of *B. diphtheriae*.
2. From 80 clinical cases of diphtheria seven serological types of bacilli have been isolated.
3. The morphological characteristics and the sugar reactions do not indicate the serological grouping of the strains.
4. Fifty-two strains were tested for virulence and 16 were found to be virulent. In two instances the strain isolated from the nose was avirulent, while the strain isolated from the throat was virulent.
5. Virulent and avirulent bacilli are not differentiated by serological tests.

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NOTES ON THE SPREAD OF BACTERIAL INFECTION.

By E. C. RHODES.

(With 2 Charts.)

IN a paper on "The Spread of Bacterial Infection" (*Journal of Hygiene*, xix. No. 4, March 22, 1921) Dr W. W. C. Topley has shown the results of many months' observation of the progress of an epidemic in a population of mice. The author thus describes the experiment: "This experiment lasted from May 21, 1919, until June 11, 1920....The epidemic started about the middle of October, 1919, and continued in the manner to be described until the conclusion of the experiment....The period May 21 to September 17 is omitted....During this time there was no indication of the active spread of infection....The whole experiment may be divided roughly into four periods. The first extends from May 21 to Sept. 17, and has been referred to already. From Sept. 18 and onwards, only normal mice were added to the cage. From this date until Jan. 5, 1920, the mice were added in such a way as to keep the total number in the cage roughly constant. From Jan. 6 to April 27, 1920, three normal mice were added each day, except on two occasions when none was added. The number of mice in the cage during this period varied from day to day, reaching a maximum immediately before each considerable wave of mortality, and falling to a minimum just before its cessation. During the final period from April 28 to June 11, 1920, two normal mice were added daily instead of three."

The author then shows that during the period when constant daily additions of normal mice are made to the population, the form of the population curve (population against time) is wavy, and exhibits a periodicity of about 40 days. He further shows that those mice introduced into the cage, when the cage population is at a maximum, have a small survival time, and those mice introduced when the cage population is at a minimum have a large survival time. The paper proceeds to discuss other experiments.

The present writer has been concerned with the first experiment and in particular with that part of this experiment from Jan. 6 to April 27, 1920, when three normal mice were added daily to the cage. He has endeavoured to get some indication of the variations in the power of the population to infect new mice, by considering the actual daily constitution of the population, according to the number of days' residence in the cage of individual mice of the population. Fortunately, in Topley's paper there is a complete history of

each mouse forming part of the population of the cage; that is to say, we know its date of entry and its date of death. This statement is true, with the exception of a few cases, cases where mice have disappeared, probably eaten by the others. Thus when one actually traces the progress of individual mice, one finds a certain number whose death is not recorded. For this reason certain figures which will be given here for the cage population will be found to differ from Topley's figures, but the *sequence* of the figures is the same as with those of Topley. When the mice are shown in a distribution according to survival time it is seen that the mode of the distribution is at ten days, and the range of the distribution is about 30 days. Actually there are one or two cases of mice who have survived much more than 30 days, and Topley in his work rejects these cases; the present writer will do the same.

To get the number of mice alive on any day of the period under discussion, and the number of days each mouse had formed part of the population the writer traced, through the records given by Topley, all the mice who were in the cage previous to this day, with the exceptions mentioned above. In this way the cage population, distributed according to the time of sojourn in the cage, was obtained for each day from Jan. 26 to April 28 inclusive. In forming these totals, the deaths in the cage reported as on April 27, say, are supposed to have occurred during that day, and the population given for April 28 is supposed to be that at the beginning of the day. Similarly, for the other days in the series. The series starts at Jan. 26, because previous to that date there are alive in the cage, mice which had been put in before the three-per-day manner of addition commenced on Jan. 6.

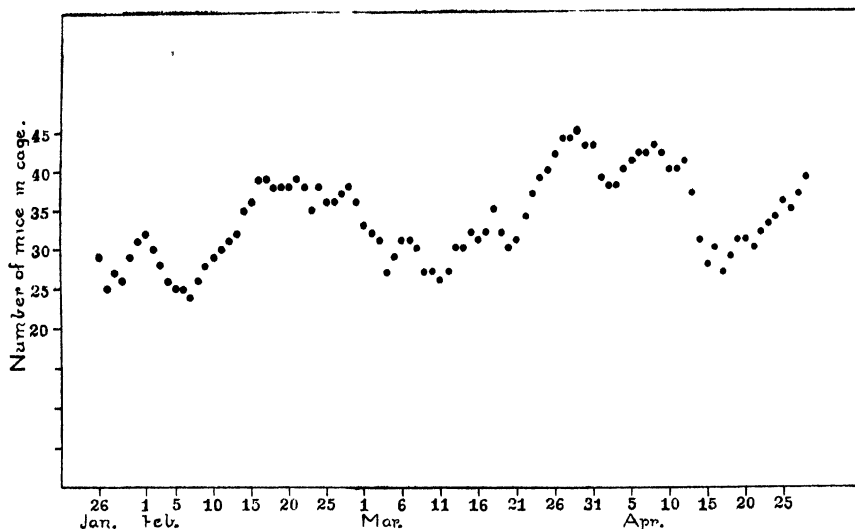
Table I.

No. of days in cage		TABLE 1																											Total	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27		
Feb. 10		3	3	3	3	3	3	3	2	.	2	.	1	1	.	2	29
Apr. 9		3	3	3	3	3	3	3	3	2	3	3	.	.	1	.	3	1	.	1	.	.	1	1	.	1	.	1	.	12

Table I shows the distribution of the population at the beginning of two days (Feb. 10 and April 9) according to the length of time the mice had been in the cage. Thus on Feb. 10 there were three mice which had been in the cage one day, three had been in two days, three three days, and so on.

Chart 1 shows the alteration in the total population with time. The periodic character of the curve is indicated in this figure. For purposes of showing the alteration in the constitution of the population, it was thought better to group those days with 24 and 25 mice together; similarly, 26, 27; 28, 29; and so on. In this way we should get a better idea of the average constitution of the population when this population was a minimum, when it was increasing and decreasing, and when it was a maximum. A typical example will illustrate what was done.

Notes on the spread of Bacterial Infection



(Chart 1. Population change with time.

Table II. *Population 26 and 27 mice.*

		Number of days in cage																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
No. of Mice	0	1	1	2	2	7	5	7	8	9	10	10	9	10	10	9	10	10	9	10	10	10	10	10
	1	1	2	2	5	6	1	3	2	2	1	.	.	1	.	.	1	.	.	1
	2	.	.	.	2	1	2	1	3	4	2	1	2	1	1
	3	10	9	8	7	8	8	7	4	2	1	1	.	1

Table II shows that on each of the ten days when the population was 26 or 27, three of the mice had been in the cage one day; on one day there were three mice which had been in the cage ten days; on two days there were two mice which had been ten days; on five days there was one mouse which had been in the cage ten days; on two days there were no mice which had been in the cage exactly ten days; on all ten days there were no mice which had been in the cage 20 days, and so on. The fact that the vertical totals in this table are not always ten, is due to the two days during the period considered when no mice at all were added to the cage.

From this table we may obtain the average number of mice of 1, 2, 3... days' sojourn in the cage contributing to a total of 26.5 mice. Similar tables made up for other populations will lead finally to Table III, where the results are shown. This table gives the constitution of the cage for totals ranging from 24 to 45, in groups 24, 25; 26, 27; 28, 29; etc., as indicated. Looking at this table, we seen that roughly all the populations on different days have the same number of mice which have been in the cage 1, 2, 3, 4, 5, 6 days, and that the difference between the total populations on two days is caused by differences in the numbers which have been in the cage seven or more days. This fact is brought out more easily in Table IV, formed from Table III, by grouping the various items together.

Table III. *Showing the Mice forming part of the population according to the length of time they have been in the cage.*

Total population	24, 25	26, 27	28, 29	30, 31	32, 33	34, 35	36, 37	38, 39	40, 41	42, 43	44, 45
Number of days in cage	1	3.00	3.00	3.00	2.94	3.00	3.00	3.00	3.00	3.00	3.00
	2	3.00	3.00	3.00	2.94	2.78	3.00	2.93	3.00	3.00	3.00
	3	3.00	3.00	3.00	2.89	2.78	2.83	2.89	2.93	3.00	2.86
	4	2.67	2.78	2.88	2.83	2.67	2.67	3.00	2.86	3.00	2.86
	5	2.67	2.89	2.12	2.78	3.00	2.67	2.67	2.79	3.00	2.71
	6	2.33	2.80	3.00	2.41	2.67	2.83	2.78	2.50	2.83	2.86
	7	1.75	2.67	2.50	2.59	2.78	2.33	2.89	2.43	2.33	3.00
	8	1.25	2.00	2.14	2.50	2.62	2.67	2.56	2.36	2.00	3.00
	9	1.50	1.60	1.43	2.29	2.00	2.67	2.44	2.36	2.67	2.43
	10	1.50	1.20	1.38	1.59	2.00	1.83	2.56	2.43	2.17	2.28
	11	1.00	1.10	.75	1.24	2.00	2.20	1.89	2.00	2.00	1.86
	12	1.75	.50	.88	.61	1.57	1.83	1.67	1.64	2.50	1.28
	13	.50	.80	.88	1.00	.78	1.20	1.33	1.57	1.83	1.86
	14	.75	.40	.75	.59	.56	.67	1.12	1.57	1.00	1.71
	15	.	.20	.62	.50	.33	1.00	1.11	1.00	.83	1.28
	16	.	.10	.12	.44	.78	1.00	.12	1.31	.17	1.14
	1744	.22	.33	.67	.92	1.00	.86
	1817	.33	.17	.11	.77	1.00	.71
	19	.	.10	.	.11	.	.33	.11	.46	1.00	.50
	20	.	.	.38	.	.	.33	.11	.31	.50	.86
	2111	.	.	.11	.38	.33	.43
	22	.	.10	.	.11	.	.	.11	.14	.17	.57
	23	.	.	.25	.06	.	.	.11	.08	.17	.33
	2411	.	.	.12	.	.17	.50
	25	.	.10	.	.0608	.17	.43
	26	.	.	.12	.06	.	.	.12	.08	.33	.14
	2706	.1114
	280620	.
	290620	.
	3011
No. of days	4	10	8	18	9	6	9	14	6	7	3

Table IV.

Population	24, 25	26, 27	28, 29	30, 31	32, 33	34, 35	36, 37	38, 39	40, 41	42, 43	44, 45
No. of mice	$\left\{ \begin{array}{l} \text{less} \\ \text{than} \\ 7 \text{ days} \end{array} \right\}$	16.67	17.47	17.00	16.09	16.89	17.00	17.34	17.00	17.83	17.29
		10.00	10.87	12.20	14.76	16.19	18.56	19.25	21.89	22.74	24.67
	$\left\{ \begin{array}{l} 7 \text{ days} \\ \text{and} \\ \text{more} \end{array} \right\}$										
		10.00	10.87	12.20	14.76	16.19	18.56	19.25	21.89	22.74	24.67

This table shows that the increase in the number of mice (or the decrease) in the cage at any time, is roughly due to an increase (or a decrease) in the number of those which have been in the cage seven days or more. Now Topley observed that mice added to the cage survived longer or shorter periods according to the time they were introduced. We can confirm this fact for the period we are considering, in a rough manner, by means of the following table (V):

Table V. *Showing the alterations of average survival time of mice added to the cage, according to the time of addition.*

Week com- mencing	Jan. 28	Feb. 4	Feb. 11	Feb. 18	Feb. 25	Mar. 3	Mar. 10	Mar. 17	Mar. 24	Mar. 31	Apr. 7	Apr. 14	Apr. 21
Average survival time (days)	11.6	12.2	11.6	10.6	9.7	11.0	13.3	14.8	11.6	11.0	9.9	20.0	14.8
Average number in cage	29.0	26.1	34.6	37.7	35.4	29.4	29.0	33.0	42.4	40.1	40.7	29.4	33.9

Notes on the spread of Bacterial Infection

This table was obtained by taking the average time of survival of mice added in the weeks, Jan. 28 to Feb. 3, Feb. 4 to Feb. 10, and so on, during the period under review. Similarly, the bottom row indicates the average daily population in these weeks. Chart 2 shows the variation in the average survival

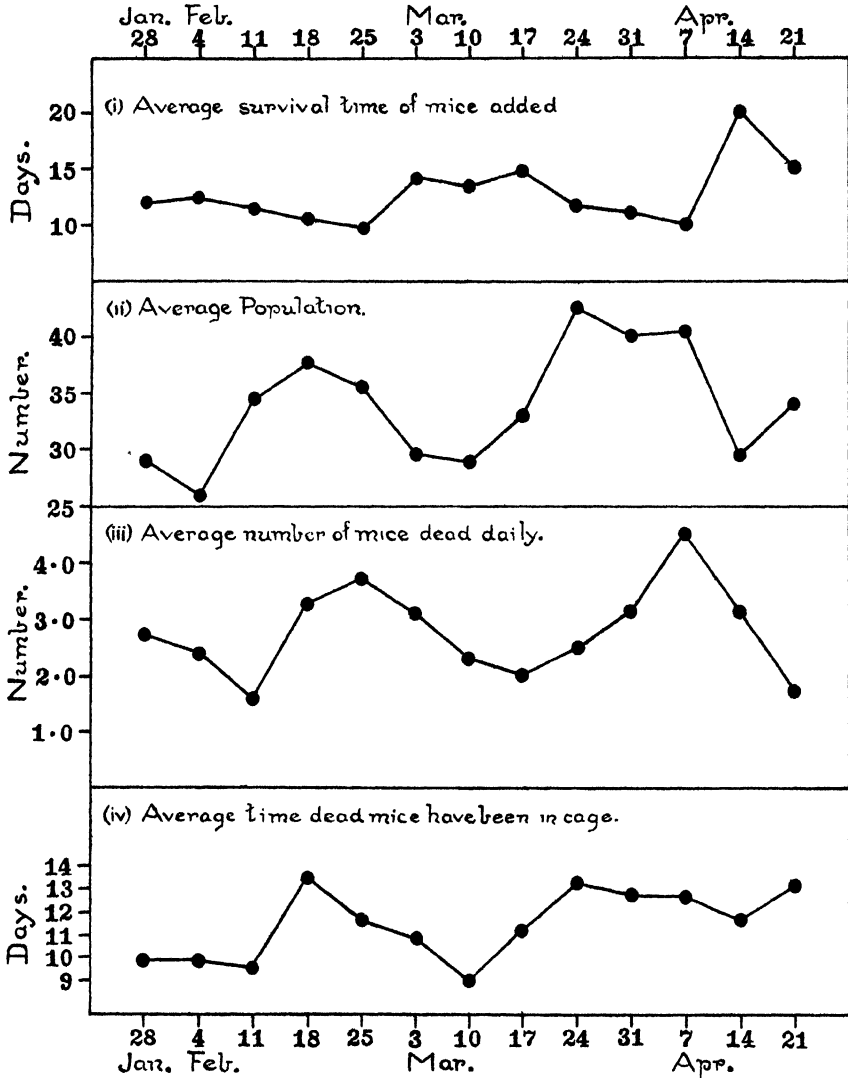


Chart 2.

time of mice added to the cage, and three other diagrams for comparison, all of which bring out the periodicity, viz.: The average daily population, the average daily deaths, and the average time in the cage of the daily batches of dead. At present, all we are concerned with are the two upper diagrams, which clearly indicate the fact that the average survival time introduced on any day

bears some relationship to the number in the cage at or about the time when the mice form part of the population. Thus we conclude that when the number of mice forming the population is large there is some peculiarity appertaining to the population, which causes mice introduced at that time to die quickly; and when the number of mice is small this quality is missing and the mice live longer. We have observed that the difference between the constitutions of the populations when maximum and minimum lies in the presence of many or few mice who have been in the cage seven or more days, and this fact suggests the possibility that the quality to convey the disease to new mice in a virulent form is related to the presence of many mice which have themselves been exposed to infection seven or more days.

This fact may also be presented in another way, thus: Since the number of mice which have formed part of the population for 1, 2, 3, 4, 5, 6 days is practically the same during the whole time of the experiment, we may say that the variations in the survival times which are observed, are not related to the presence of mice in the cage of less than seven days' sojourn therein.

It would naturally be expected that in this experiment where the same number of new mice are added to the population each day, there would be more "older" mice when the total population is large than when the total population is small; but the writer is desirous of emphasising that there is always practically the same number of mice which have formed part of the population for periods ranging from one to six days.

In Chart 2, (iii) and (iv) have been given, because they help to bear out the conclusions obtained as to periodicity by Topley, and (iv) in addition supports Table III. From this table, we see that when the daily total changes from (say) 42 to 43, the dead mice on the day of the change will come from that part of the population whose time of sojourn is from 7 to 25 days (roughly); and when the daily total changes amongst the smaller numbers (24-25), the dead will be of those whose time in the cage has been 7 to 14 days. This is borne out by Chart 2 (iv) which shows that the average time of sojourn in the cage of the daily dead is greatest when the population is greatest, and least when the latter is least.

It was not thought of advantage to pursue the investigations further—it would be of interest to compare exactly the constituent populations day by day with the survival times of the mice introduced, but the numbers involved are so few, that there is danger of obtaining results which are really worthless, if finer methods of treatment are used.

DURATION OF PASSIVE IMMUNITY.

By A. T. GLENNY AND BARBARA E. HOPKINS.

(*From the Wellcome Physiological Research Laboratories.*)

PART II.

(With Charts III—XII.)

(Continued from Vol. XXI, No. 2.)

IN Part I of this paper we traced the rate of disappearance of diphtheria antitoxin obtained from a horse, when injected intravenously into normal rabbits and into rabbits previously sensitised by the subcutaneous injection of small volumes of horse serum. We described the curve of antitoxin content as falling into three phases: Phase A, a rapid loss of 50 per cent. during the first 24 hours, Phase B, a period of slow elimination lasting seven or eight days in normal rabbits during which about 25 per cent. of the amount present each day is lost by the next day, and Phase C, a period of accelerated loss apparently due to the formation of precipitin in response to the antigenic stimulus of the injection of horse serum. It would be expected *a priori* that if heterologous serum were injected into a number of normal animals, *e.g.* rabbits, all the phenomena which followed would be practically identical in the different animals. Early in our work it became obvious that considerable differences occurred in the response in different animals; these differences, particularly in regard to the duration of Phase B and the duration and intensity of Phase C, are set out in full detail in the tables in this paper and various explanations of the differences are considered. Observations on two atypical normal rabbits 42 and 61 are recorded in Table V. The rate of elimination of horse serum from rabbit 42, as indicated by antitoxic content, differs considerably from that of other normal rabbits 41, 49, and 50, recorded in Part I, Tables I and II. Reference to Table V and Curve 11 on Chart III shows that Phase C is succeeded by a fourth phase of slow elimination indicating apparently that rabbit 42 did not produce sufficient precipitin to eliminate all the precipitinogen injected. In contrast to this rabbit, rabbit 61 (Table V and Curve 12 on Chart III) responded earlier with a more rapid excretion probably due to a more rapid production of precipitin than did the normal rabbits previously recorded.

The duration of Phase B must be taken as the Latent Period between the injection of precipitinogen and the production of precipitin; the rapidity of accelerated loss during Phase C must be taken as an indication of the rapidity

Table V.

Showing the differences between the responses of two normal rabbits to an intravenous injection of 0.5 c.c. unconcentrated horse serum containing 750 units diphtheria antitoxin.

Time interval	Antitoxic value in units per c.c.		Percentage daily loss		
	Rabbit 42	Rabbit 61	Rabbit 42	Rabbit 61	
15 minutes	6.5	10.5			
1 day	—	4.0	—	61.9	
2 days	3.0	3.0	—	25.0	Phase B Average 27.1
3 "	3.0	—	0.0	—	
4 "	1.8	1.5	40.0	29.3	
5 "	1.5	0.8	16.7	46.7	Phase C Average 77.5
6 "	1.1	0.14	26.7	82.5	
7 "	0.9	0.02	18.2	85.7	
8 "	—	0.001	—	95.0	
9 "	0.22	0.0005	50.6		Phase C Average 57.5
10 "	0.08	—	63.7		
11 "	0.035	—	56.2		
12 "	0.016	—	54.3		
13 "	0.006	—	62.5		
14 "	0.004	—	33.3		Phase D Average 21.5
15 "	—	—	—		
16 "	0.002	—	29.3		
17 "	—	—	—		
18 "	0.001	—	29.3		
19 "	—	—	—		
20 "	0.001	—	0.0		
21 "	—	—	—		
22 "	—	—	—		
23 "	0.0006		15.6		
24 "	—				
25 "	—				
26 "	<0.0005				

of formation of precipitin, *i.e.* of immunity response to the injection of horse serum. The difference between the normal rabbits so far recorded is suggestive, therefore, of differences in degree of natural immunity. Rabbit 61, apparently more highly immune naturally than the average rabbit, responds to the injection of an antigen by a more rapid and intense production of antibody, than do the majority of normal rabbits. Similarly rabbit 42 must be regarded as less highly immune naturally, because the same stimulus in this rabbit produced a smaller production of antibody than in other normal rabbits.

It was this conception that led us to suggest that the 50 per cent. loss during Phase A was due to natural precipitin. Later considerations have led

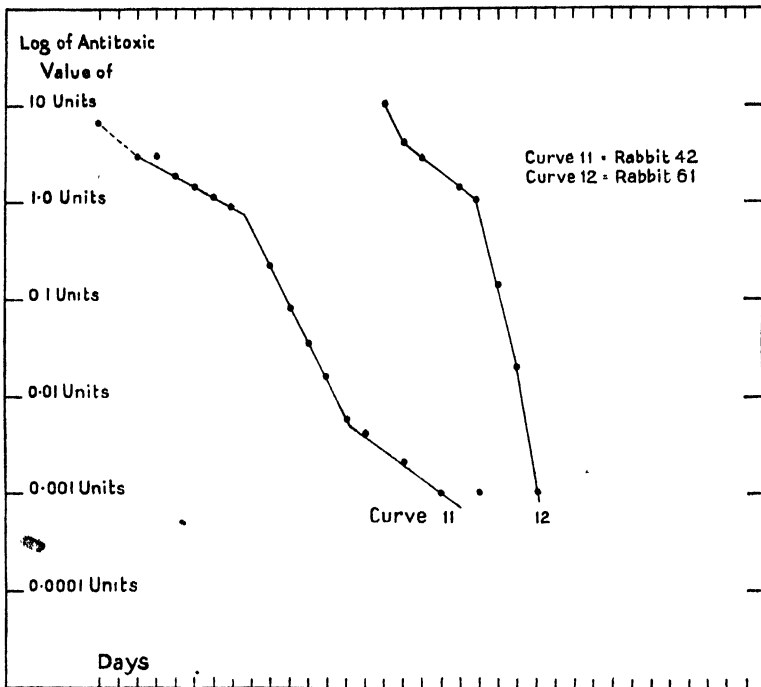


Chart III.

us to abandon this idea. At first the variation in percentage loss during Phase A was taken to be of marked significance. Although no bleeding was taken from rabbit 42 on the day following injection, yet the shape of the curve of antitoxic content for this rabbit suggests that the loss during Phase A was decidedly smaller than the average loss of 50 per cent. It therefore appeared that rabbits 42 and 61, regarded as the least and the most highly immune rabbits, exhibited the smallest and the greatest loss respectively during Phase A. Later considerations, however, have shown that the antitoxic value recorded for rabbit 42, 15 minutes after injection, was lower than the value observed in other rabbits of similar weight. The connection between the weight of the animal and the amount of antitoxin detectable in the serum 15 minutes after

an intravenous injection of a given amount of antitoxin will be considered in a later part. Again, rabbit 61 gave a value nearly 30 per cent. higher than the theoretical value for the 15 minutes' reading, but if the actual 24 hours' reading be compared with the theoretical 15 minutes' reading it shows a loss of 50.9 per cent., thus falling into line with other normal rabbits. It is probable, therefore, that these differences in percentage loss during Phase A are due to some unexplained error in testing and are of no significance. There remain, however, the apparent differences in natural immunity.

The five normal rabbits under review were again injected with horse serum containing diphtheria antitoxin, in order to determine whether the initial differences in immunity response continued. Rabbits 50 and 61 were re-injected 10 and 11 days respectively after the first injection; the results of re-injection are recorded in Table VI and Curves 13 and 14 on Chart IV. Rabbit 61 was not bled 15 minutes after injection, but the value taken has been calculated according to the weight of the animal. The results obtained failed to show any differences in immunity response to the second injection because the usual phases are here masked by the greater loss caused by excess precipitin still present or still being formed in response to the initial stimulus; rabbit 61, however, had eliminated all antitoxin earlier than rabbit 50.

Table VI.

Showing the differences between the responses of rabbits 50 and 61 when re-injected after a short interval with 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Rabbit	Units of antitoxin remaining		Percentage daily loss	
	50	61	50	61
No. of days between 1st and 2nd injection	10	11	—	—
Time interval				
15 minutes	8.0	(8.02)	65.6	59.4
1 day	2.75	3.25	54.6	38.5
2 days	1.25	2.0	82.4	60.0
3 "	0.22	0.8	86.4	96.2
4 "	0.03	0.03	85.0	95.0
5 "	0.0045	0.0015	66.7	
6 "	0.0015	—	20.0	
7 "	0.0012	0.0005	50.0	
8 "	0.0006			

Rabbits 41, 42 and 49 received their second injection of antitoxic horse serum 7–10 weeks after their first injection. It will be seen from Table VII and Curves 15, 16 and 17 on Chart IV that rabbit 42 again shows a smaller

Duration of Passive Immunity

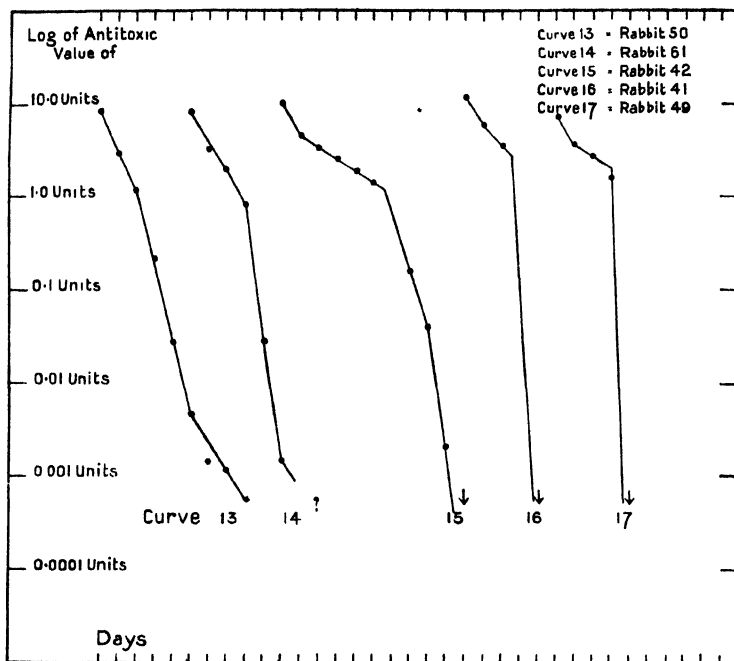


Chart IV.

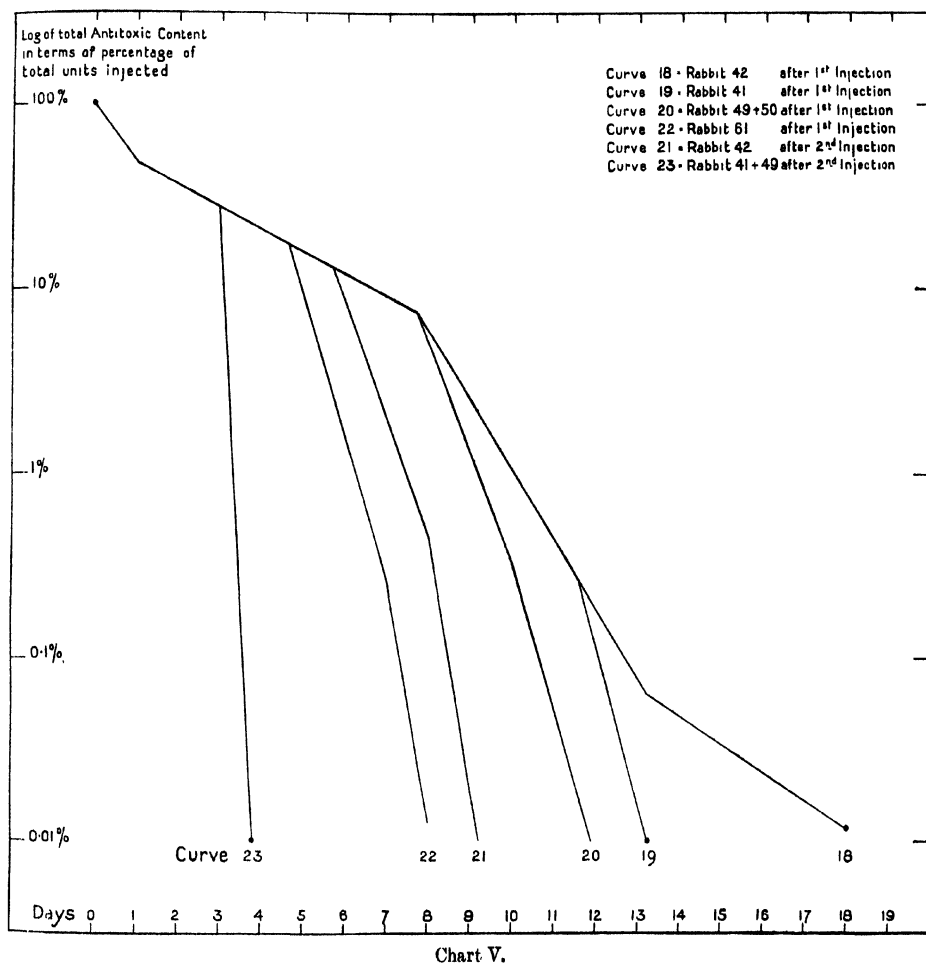
Table VII.

Comparing responses of rabbits 41, 42, and 49 when re-injected after a considerable space of time with 0.5 c.c. unconcentrated horse serum containing 750 units diphtheria antitoxin.

Rabbit	Units of antitoxin remaining			Percentage daily loss		
	42	41	49	42	41	49
Number of days between 1st and 2nd injection	72	47	62	—	—	—
Time interval						
15 minutes	10.0	12.0	6.5	52.5	54.2	46.2
1 day	4.75	5.5	3.5	31.5	31.8	21.4
2 days	3.25	3.75	2.75	23.1	26.3	41.8
3 "	2.5	—	1.6	28.0	(c. 99.0) Phase C	
4 "	1.8	<0.0005	<0.0005	22.2	(c. 99.0) Phase C	
5 "	1.4	—	—	—		
6 "	—	—	—	(86.2)		
7 "	0.16	—	—	75.0	Phase Q	
8 "	0.04	—	—	95.0	Average	
9 "	0.002	—	—	75.0	78.7	
10 "	<0.0005	—	—	—		

immunity response. Rabbits 41 and 49 were regarded as average normal rabbits according to their responses to the first injection of horse serum. The responses of these rabbits to a second injection agree closely with those obtained with sensitised rabbits in Part I (see Tables III and IV, Curves 4-10).

A comparison between Curves 12 and 15 shows that an untreated normal rabbit number 61, may exhibit the same degree of apparent immunity as



another rabbit, number 42, that had already received an initial sensitising injection.

Chart V has been compiled in order to show more clearly the points of similarity and difference between the various curves of these five rabbits. This chart differs from the previous ones in recording the total antitoxic content of each rabbit on different days rather than the antitoxic value per c.c.; this

total content has been calculated by multiplying the antitoxic value by the available serum volume, taken as a definite fraction of the body weight. In this way the values for the different rabbits are comparable, and we have drawn to represent Phases A and B for all the rabbits a single standard curve from which the curve representing Phase C for each individual rabbit branches off. By taking the available serum volume as 44.2 c.c. per kilo. of body weight the average antitoxin content immediately after injection of the five normal rabbits recorded in Tables I and V and the three re-injected rabbits recorded in Table VII becomes equal to 750 units, the amount injected. It is not suggested that the course of elimination from each rabbit followed exactly the course depicted by the curves, but the general comparison of one rabbit with another is clearly indicated. Phase B ends, for most of the normal rabbits, between the seventh and the eighth day after injection, but differences occur in the slope of the curves representing Phase C. The general interpretation of this chart is that after a single injection of the same volume of horse serum into five normal rabbits the latent period before the formation of precipitin was 7-8 days in four rabbits, but the amount formed varied; the immunity response was least in rabbit 42 represented by Curve 18, more in rabbit 41 (Curve 19) and again more in rabbits 49 and 50 (Curve 20 represents rabbit 49 along its full length and rabbit 50 until re-injected). The fifth normal rabbit number 61 (Curve 22) showed a latent period of just under five days and the intensity of the immunity response, indicated by the steepness of the section of curve for Phase C, was greater than that of the other four rabbits. Upon again injecting rabbits 41 and 49, the curve of antitoxic content (curve 23) is moved well to the left showing a latent period of only three days followed by a rapid production of precipitin as indicated by the steepness of the section of curve representing Phase C. Rabbit 42 upon re-injection also showed an increased response (Curve 21). While the various curves following the first injection of horse serum into these rabbits show that normal rabbits differ in responsiveness to an injection of horse serum, the position of the curve for rabbit 61, standing as it does to the left of the curve following the second injection into rabbit 42, is strongly suggestive that this difference in responsiveness is a difference in degree of natural immunity.

If this view were correct and some normal rabbits became during life more immune to horse serum than others, young rabbits would probably show a lower average degree of immunity than older rabbits that have had more opportunity of acquiring an artificial immunity. The ages of the five normal rabbits were not recorded, but at the time of their first injection they each weighed between 1800 and 2050 grams.

A number of very young rabbits were injected intravenously with antitoxic horse serum and the curves of their antitoxic content traced. Tables VIII and IX and Chart VI give the results obtained with four rabbits from the same litter injected at various ages. These rabbits did not show a uniform progressive immunity to horse serum in accordance with their age at the time of injection.

Table VIII.

Showing the antitoxic value of the blood of four normal rabbits of the same litter, at different intervals of time, after the injection of 0.5 c.c. unconcentrated horse serum containing 750 units of diphtheria antitoxin.

Rabbit	76	79	86	93
Weight at time of injection	510	740	680	1020
Age at time of injection	7 wks.	11 wks.	13 wks.	16 wks.
Time interval	Antitoxin value in units per c.c.			
15 minutes	35.0	23.0	25.5	15.5
1 day	17.0	11.0	11.0	7.5
2 days	12.0	7.5	8.0	4.75
3 "	6.5	6.5	6.0	—
4 "	5.5	5.5	4.75	2.75
5 "	4.75	3.75	3.0	2.25
6 "	—	—	—	0.8
7 "	3.5	0.8	1.62	0.45
8 "	1.87	0.25	1.25	—
9 "	1.5	0.07	0.9	0.12
10 "	1.25	0.011	0.7	—
11 "	0.8	0.003	0.35	—
12 "	0.5	0.0014	0.22	0.015
13 "	—	—	—	0.008
14 "	—	0.0006	0.14	0.005
15 "	0.14	0.0005	0.11	0.0025
16 "	0.09	<0.0005	0.08	0.001
17 "	0.055	—	0.045	—
18 "	0.05	—	0.035	0.001
19 "	0.035	—	0.022	0.0005
20 "	—	—	—	—
21 "	0.018	—	0.015	—
22 "	0.012	—	0.011	—
23 "	0.009	—	0.0055	—
24 "	—	—	—	—
25 "	0.0055	—	0.0045	—
26 "	0.003	—	0.0040	—
27 "	—	—	—	—
28 "	0.0016	—	—	—
29 "	—	—	—	—
30 "	0.0010	—	0.0025	—
31 "	0.0010	—	—	—
32 "	0.0006	—	0.002	—
33 "	0.0005	—	—	—
34 "	—	—	—	—
35 "	—	—	—	—
36 "	—	—	—	—
37 "	—	—	—	—
38 "	—	—	0.0015	—
39 "	—	—	0.0010	—
40 "	—	—	—	—
41 "	—	—	0.0005	—
42 "	—	—	—	—

Two of the rabbits showed such a low degree of immunity that the existence of Phase C in Curves 24 and 26 is difficult to demonstrate. These two rabbits 76 and 86, aged 7 and 13 weeks and weighing 510 and 690 grams at the time of injection, retained some antitoxin for over four weeks; their curves, if plotted on the standard curve on Chart V would be well to the right of Curve 18. On the other hand, two others of the same litter, 79 and 93, aged 11 and 16 weeks and weighing 740 and 1020 grams, were far more responsive. Curves 25

Table IX.

Showing the percentage daily loss in antitoxic value of the blood of four normal rabbits of the same litter, at different intervals of time, after the injection of 0.5 c.c. unconcentrated horse serum containing 750 units of diphtheria antitoxin.

Time interval	Rabbit 76		Rabbit 79		Rabbit 86		Rabbit 93	
0-1 day	51.4	Phase A	52.2	Phase A	56.8	Phase A	51.6	Phase A
1-2 days	29.4		31.8		27.3		36.7	
2-3 "	45.8	Phase B	13.3	Phase B	25.0	Phase B	—	Phase B
3-4 "	15.4		15.4	Average	20.8		23.9	Average
4-5 "	13.6		31.8	23.1	36.8		18.2	26.3
5-6 "	—	Average	—		—	Average	64.4	
6-7 "	14.2		53.8		26.5		43.7	
7-8 "	46.6		68.7		22.8		—	
8-9 "	19.8	Phase C	72.0		28.0	Phase C	48.4	
9-10 "	16.7		84.3	Phase C	22.2		—	Phase C
10-11 "	36.0		72.7	Average	50.0		—	Average
11-12 "	37.5	Average	53.3	67.5	37.1	Average	50.0	50.1
12-13 "	—		—		—		46.7	
13-14 "	—		35.5		20.2		37.5	
14-15 "	34.6	Phase D	—		21.4	Phase D	50.0	
15-16 "	35.7		—		27.3		60.0	
16-17 "	38.9		—		43.7		—	
17-18 "	9.1	Average	—		22.2	Average	—	
18-19 "	30.0		—		37.1		—	
19-20 "	—		—		—		—	
20-21 "	28.3	Phase D	—		17.4	Phase D	—	
21-22 "	33.3		—		26.7		—	
22-23 "	25.0		—		50.0		—	
23-24 "	—	Average	—		—	Average	—	
24-25 "	21.8		—		9.5		—	
25-26 "	45.4		—		11.1		—	
26-27 "	—	Phase D	—		—	Phase D	—	
27-28 "	27.0		—		—		—	
28-29 "	—		—		—		—	
29-30 "	21.0	Average	—		11.1	Average	—	
30-31 "	—		—		—		—	
31-32 "	—		—		10.5		—	

and 27 showing the logs of the antitoxic values of these two rabbits exhibit irregular features when compared with standard curves on Chart V. The early appearance of Phase C would place these curves between that representing the first injection into rabbit 61, and that representing the second injection into rabbit 42. The slope of the Phase C section of the curve, however, is less steep than that of Curves 19 and 20. The latent period was short, but the rate of production of precipitin was slow.

If the differences of responses of the different rabbits tested are due to immunity acquired since birth, then these marked differences in young rabbits from the same litter would indicate that such immunity is not acquired at all consistently, and that marked differences will be seen between the responses of different young rabbits. Unless acquired immunity is lost as easily as it is gained; older rabbits should show more frequently a high degree of immunity.

Tables X and XI record the results of injecting three old rabbits and two young rabbits with antitoxic horse serum. These three old rabbits 83, 109,

and 110 (Curves 28, 29, 30 on Chart VII) appeared highly immune and all antitoxin was lost in nine days or less.

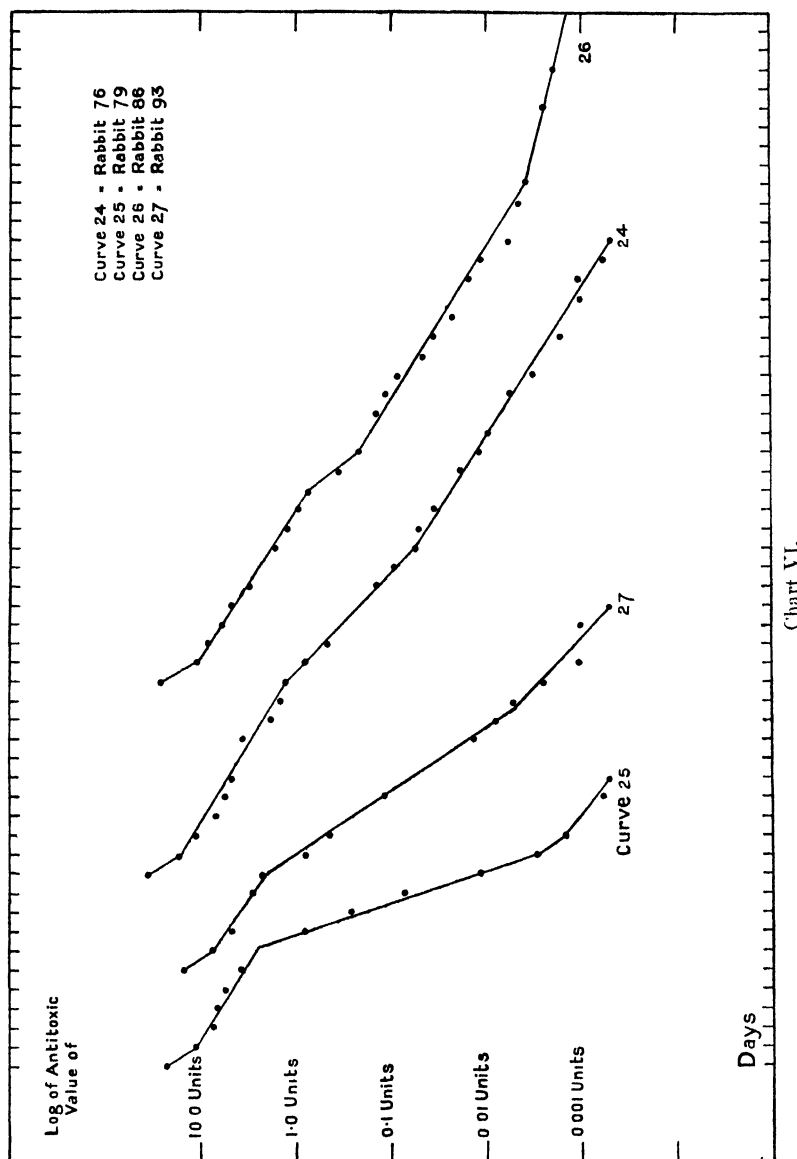


Chart VI.

The steepness of the slope of the portions of the curves representing Phase C is particularly noticeable. The formation of precipitin is obviously very rapid in old rabbits.

Of the two young rabbits, number 84 (Curves 31 and 31A) was only four weeks old and weighed only 400 grams when first injected; a moderate response

Table X.

Showing the antitoxic content of three old and two young rabbits at different intervals of time, after the injection of 0.5 c.c. unconcentrated horse serum containing 750 units of diphtheria antitoxin.

Rabbit	83	109	110	84	94
Weight in grams at time of injection	3240	2840	2810	400	910
Age	Over 2 yrs.	—	—	4 wks.	7 wks.
Time interval	Antitoxic value in units per c.c.				
15 minutes	5.25	7.0	8.0	29.0	16.0
1 day	2.75	4.5	3.75	15.5	9.5
2 days	2.0	—	—	13.0	5.75
3 "	1.62	2.5	2.25	11.0	—
4 "	1.37	1.12	1.62	8.5	3.5
5 "	1.12	0.7	0.45	6.5	2.75
6 "	—	0.035	0.0015	—	0.12
7 "	0.005	0.001	<0.001	2.75	0.004
8 "	0.0005	<0.001	—	2.25	0.002
9 "	—	—	—	1.25	0.0015
10 "	—	—	—	0.4	—
11 "	—	—	—	0.11	—
12 "	—	—	—	0.012	<0.0005
13 "	—	—	—	—	—
14 "	—	—	—	0.002	—
15 "	—	—	—	0.0006	—
16 "	—	—	—	<0.0005	—

Table XI.

Showing the percentage daily loss in antitoxic value of the blood of three old and two young rabbits at different intervals of time, after the injection of 0.5 c.c. unconcentrated horse serum containing 750 units of diphtheria antitoxin.

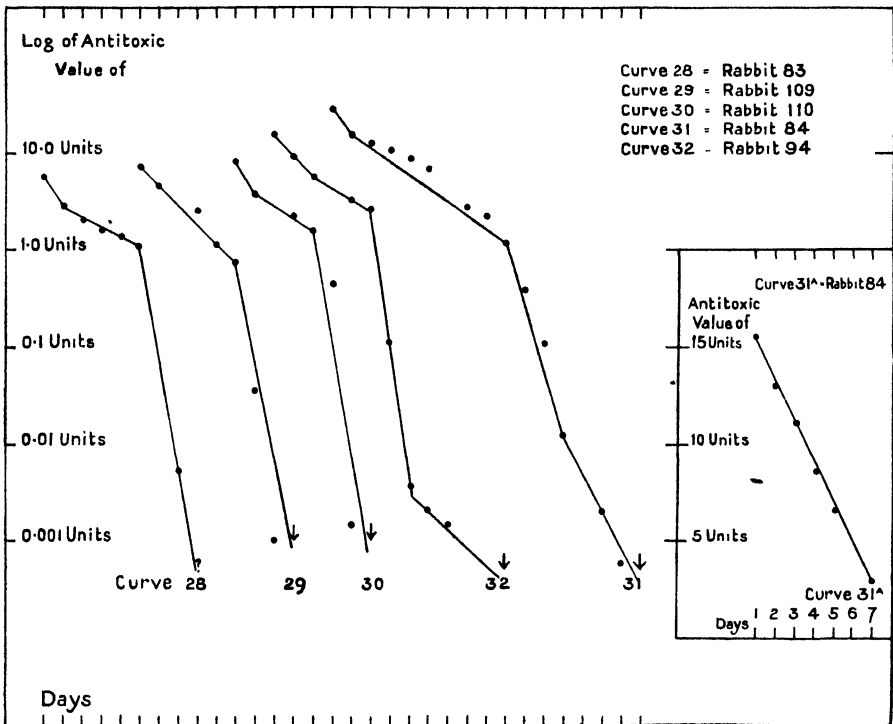
Time interval	Rabbit 83	Rabbit 109	Rabbit 110	Rabbit 84	Rabbit 94
0-1 day	47.6 Phase A	35.7 Phase A	53.1 Phase A	46.5 Phase A	40.6 Phase A
1-2 days	27.3 Phase B	— Phase B	— Phase B	16.1 Phase B	39.4 Phase B
2-3 "	19.0 Average	25.5 Average	22.5 Average	15.4 Average	— Average
3-4 "	15.4 20.0	55.2 Phase C	28.0 25.2	22.7 Phase B	22.0 Phase B
4-5 "	18.2 Average	37.5 Phase C	72.2 Phase C	23.5 Average	21.4 Average
5-6 "	— Phase C	95.0 Average	99.6 Average	—	95.6 Phase C
6-7 "	93.3 Average	97.1 71.2	85.4	35.0	96.7 Average
7-8 "	—	—	—	18.2	50.0 Average
8-9 "	—	—	—	44.4	25.0
9-10 "	—	—	—	68.0 Phase C	—
10-11 "	—	—	—	72.5 Average	—
11-12 "	—	—	—	89.1	66.6
12-13 "	—	—	—	—	—
13-14 "	—	—	—	59.2	—

resulted. This rabbit does not show the usual form of Phase B. Curve 31A has been plotted from the direct values of antitoxin content from the second to the seventh day and shows that the direct values rather than the logs of the values lie upon a straight line. A possible explanation is that although Phase B consists of a normal process of elimination of a foreign protein at a pace determined by the concentration of that protein in the blood yet there is a limit to the amount of protein that a rabbit can eliminate in a given time. Thus it is possible that so small a rabbit as 84 could eliminate in one day only

the amount of protein represented by a change in antitoxic value of 2 units per c.c. of blood.

Rabbit 94 (Curve 32), although only seven weeks old, showed a latent period of only five days, and must be regarded as highly immune for a young rabbit.

A number of other rabbits were injected with diphtheria antitoxin in an endeavour to determine further points of interest. All the rabbits previously recorded had been injected with 0.5 c.c. of unconcentrated horse serum. The effect of varying the amount of serum injected is shown in Tables XII, XIII, and Curves 31 and 34 on Chart VIII. Rabbit 71 received one-tenth the usual



amount of antitoxic horse serum, rabbit 72 received 0.5 c.c. diluted with nine times that amount of normal horse serum and rabbit 74 was injected with 10.0 c.c. of unconcentrated antitoxic horse serum representing twenty times the usual amount injected.

The three rabbits gave fairly typical curves; Phase A was exceptionally high in rabbit 71 which was injected with a small quantity of serum, and exceptionally low in rabbit 74 which was injected with twenty times the usual amount of serum. Rabbit 74 is of particular interest in that Phase A appears to extend over two days; the relatively high percentage loss for rabbit 72 between the first and the second day is also suggestive of a lengthened Phase A.

Duration of Passive Immunity

Table XII.

Showing the antitoxic content of the serum of normal rabbits at various intervals of time after intravenous injection of varying amounts of unconcentrated horse serum containing diphtheria antitoxin.

	Rabbit 71	Rabbit 72	Rabbit 74	Rabbit 75
Weight	1470	1590	2380	1190
Units of antitoxin injected	75	750	15,000	900
Volume	0.05 c.c.	5.0 c.c.*	10.0 c.c.	10.0 c.c.†
	Antitoxic value in units per c.c.			
Time interval				
15 minutes	1.325	9.5	160.0	17.0
1 day	0.55	4.5	90.0	8.5
2 days	0.45	3.25	52.0	6.0
3 "	0.3	2.75	45.0	5.0
4 "	0.22	2.25	35.0	—
5 "	0.15	1.325	22.0	2.25
6 "	0.11	0.7	—	1.75
7 "	0.09	0.25	6.5	0.8
8 "	0.07	0.14	1.62	0.22
9 "	0.03	0.08	0.14	0.04
10 "	0.011	0.033	0.009	0.005
11 "	0.0015	0.012	0.002	—
12 "	<0.0005	0.006	0.001	<0.0005
13 "	—	0.0010	—	—
14 "	—	—	<0.0010	—
15 "	—	—	—	—
16 "	—	—	—	—
17 "	—	—	—	—

* 0.5 c.c. diphtheria antitoxic serum diluted with 4.5 c.c.

† 10 c.c. of rabbit serum (74) 24 hours after the injection of unconcentrated horse serum.

Table XIII.

Showing the percentage daily loss in antitoxic value of the blood, of four normal rabbits at different intervals of time, after the intravenous injection of different quantities of diphtheria antitoxin contained in unconcentrated horse serum.

Time interval	Rabbit 71		Rabbit 72		Rabbit 74		Rabbit 75	
0-1 day	58.5	Phase A	52.6	Phase A	43.7	Phase A	50.0	Phase A
1-2 days	18.2		27.7		42.2	Av. 42.9	29.4	
2-3 "	33.3		15.4	Phase B	13.4	Phase B	16.7	Phase B
3-4 "	26.7	Phase B	18.2	Average	22.2	Average	—	Average
4-5 "	31.8	Average	41.1	25.6	37.1	24.2	32.9	25.3
5-6 "	26.7	25.3	47.1		—		22.2	
6-7 "	18.2		64.3		45.6		54.3	
7-8 "	22.2		44.0	Phase C	75.0	Phase C	72.5	Phase C
8-9 "	57.1	Phase C	42.8	Average	91.3	Average	81.8	Average
9-10 "	63.3	Average	58.7	56.7	93.5	72.2	72.9	72.9
10-11 "	86.4	68.4	63.6		77.8		—	
11-12 "	>68.7		50.0		50.0		>68.4	
12-13 "	—		83.3					
13-14 "	—		—					

Phase B was longest for rabbit 71 and Phase C shortest in the same rabbit. If these differences are significant it would indicate that a longer latent period follows the injection of a smaller amount of antigen but the rate of production of precipitin is greater, relative to the amount of precipitinogen, in the animal

injected with the smallest quantity. The other rabbit 75 recorded in this table was injected with 10 c.c. of serum taken from rabbit 74, 24 hours after the injection of horse serum. This was done to determine whether antitoxin obtained from a horse undergoes any radical alteration after injection into a rabbit, more particularly at the end of Phase A. The curve exhibited by rabbit 75 is typical of the curve of excretion of antitoxin obtained from horses showing that no such alteration has occurred.

In the next group of rabbits recorded in Tables XIV and XV, and Curves 35 to 38 on Chart IX, the diphtheria antitoxin injected was contained in

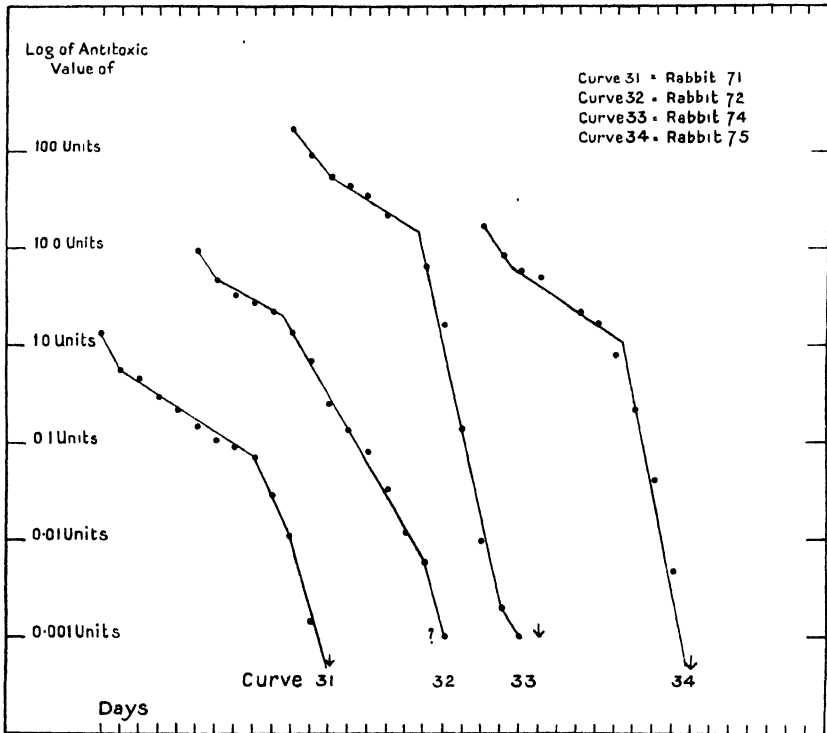


Chart VIII.

purified pseudo-globulin. This concentrated product contained about 50 per cent. more protein than the antitoxic horse serum previously used. No special precautions had been taken in the preparation of this product to render it entirely free from traces of euglobulin or of albumen. Only one rabbit, 73, was traced in detail. This rabbit received slightly more protein than the majority of rabbits already recorded and gave a typical curve. The other three rabbits were not tested until the sixth day. In all cases large quantities of protein were injected, 6 c.c. of pseudo-globulin containing from 7200 to 9000 units. The outstanding feature of the results obtained from these rabbits is the presence of Phase D. It must be remembered that we have no test for

Table XIV.

Showing the antitoxic content of normal rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in purified horse pseudo-globulin.

Rabbit	73	103	104	105
Weight	1390	1640	1930	1620
Units of antitoxin injected	1000	8400	9000	7200
Volume	0.63 c.c.	6 c.c.	6 c.c.	6 c.c.
Time interval	Antitoxic value in units per c.c.			
15 minutes	15.0	(114)*	(103)*	(100)*
1 day	6.25	—	—	—
2 days	5.5	—	—	—
3 "	4.75	—	—	—
4 "	3.5	—	—	—
5 "	—	—	—	—
6 "	1.62	13.0	12.0	7.5
7 "	—	10.0	6.5	3.75
8 "	0.11	5.75	3.25	2.25
9 "	0.0035	3.0	1.62	1.25
10 "	0.0001	0.55	0.62	0.55
11 "	<0.0005	0.055	0.3	0.17
12 "	—	—	—	—
13 "	—	0.003	0.075	0.08
14 "	—	0.0015	0.05	0.05
15 "	—	0.0015	0.015	0.015
16 "	—	0.0015	0.008	0.015
17 "	—	0.001	0.007	0.01
18 "	—	<0.001	0.006	0.008
19 "	—	—	—	—
20 "	—	—	0.003	0.0045
21 "	—	—	—	—
22 "	—	—	0.002	0.004
23 "	—	—	—	—
24 "	—	—	0.0015	0.003
25 "	—	—	—	—
26 "	—	—	—	—
27 "	—	—	0.0015	0.0015
28 "	—	—	—	—
29 "	—	—	0.001	<0.001
30 "	—	—	—	—
31 "	—	—	<0.001	—

* Values calculated according to weight of animal.

the total absence of antitoxin, but it is possible to say that the amount of antitoxin present is less than one thousandth of a unit per c.c. and in many of our earlier experiments we were able to detect with comparative certainty the presence or absence of one two-thousandth part of a unit per c.c. The shape of the curve for any rabbit after the antitoxic content had fallen below this level is impossible to determine. With the majority of rabbits this level bore roughly the same relationship to the total amount of antitoxin present as in the case of rabbit 73 in the table under review, *i.e.*, about one fifteen-thousandth of the total antitoxin injected. Rabbits 103, 104, 105 injected with 7–10 times the usual amount of antitoxin could be followed out over a longer range than other rabbits, the least amount detectable being approximately

Table XV.

Showing the percentage daily loss in antitoxic value of the blood of normal rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in purified horse pseudo-globulin.

Time interval	Rabbit 73	Rabbit 103	Rabbit 104	Rabbit 105
0-1 day	58.3	Phase A		
1-2 days	12.0			
2-3 "	13.6	Phase B		
3-4 "	26.3	Average		
4-5 "	—	21.0		
5-6 "	32.0			
6-7 "	—	Phase C	23.0	Phase B
7-8 "	73.8	42.5	45.8	50.0
8-9 "	96.8	47.8	50.0	40.0
9-10 "	71.4	80.7	50.0	44.4
10-11 "	—	81.7	61.7	56.0
11-12 "	—	90.0	51.6	69.1
12-13 "	—	—	64.8	—
13-14 "	—	76.7	50.0	31.4
14-15 "	—	50.0	33.3	37.5
15-16 "	—	(29.3)	70.0	70.0
16-17 "	—	(20.6)	Average	(45.2)
17-18 "	—	33.3	27.7	33.3
18-19 "	—	—	12.5	20.0
19-20 "	—	—	14.3	—
20-21 "	—	—	29.3	25.0
21-22 "	—	—	18.3	5.7
22-23 "	—	—	—	—
23-24 "	—	—	13.4	13.4
24-25 "	—	—	—	—
25-26 "	—	—	—	—
26-27 "	—	—	(5.5)	20.5
27-28 "	—	—	—	—
28-29 "	—	—	18.3	—
29-30 "	—	—	—	—
30-31 "	—	—	—	—

one hundred-thousandth of the total antitoxin injected. The comparable level of least detectable amounts, one fifteen-thousandth, was passed for these three rabbits between the twelfth and the seventeenth day before the existence of Phase D could be determined with certainty. This suggests the possibility of Phase D being of frequent occurrence although infrequently detected. On the other hand it must be pointed out that in the next tables another rabbit is recorded that was injected with 7500 units of antitoxin without exhibiting signs of Phase D while rabbit 74 in Table XVI received 15,000 units and Phase D was not detected. The most probable explanation is that when relatively large amounts of serum are injected the amount of precipitin produced is insufficient to eliminate all the precipitinogen.

Tables XVI and XVII and Curves 39, 40, 41 on Chart X record the results of injecting three rabbits from the same litter at the same time with different amounts of diphtheria antitoxin contained in purified horse globulin. The exceptionally high figures for percentage loss during Phase A are probably due to loss by withdrawal of blood; these three rabbits were all bled seven times

in the first 24 hours after injection in an endeavour—to be reported later—to explore more fully Phase A. The total amount of blood withdrawn during this period was from 20 to 30 per cent. of the total blood content of the rabbits. The extent of loss during Phase A is again in the same order, *i.e.*, the greatest loss occurs in the rabbit receiving the smallest injection. The short duration of the presence of any detectable antitoxin in rabbit 106 and the long duration in the rabbit 108 shows an artificial difference because the least detectable amount of antitoxin is relatively smaller in proportion to the amount injected in the rabbit receiving the large injection. These tables and the two preceding

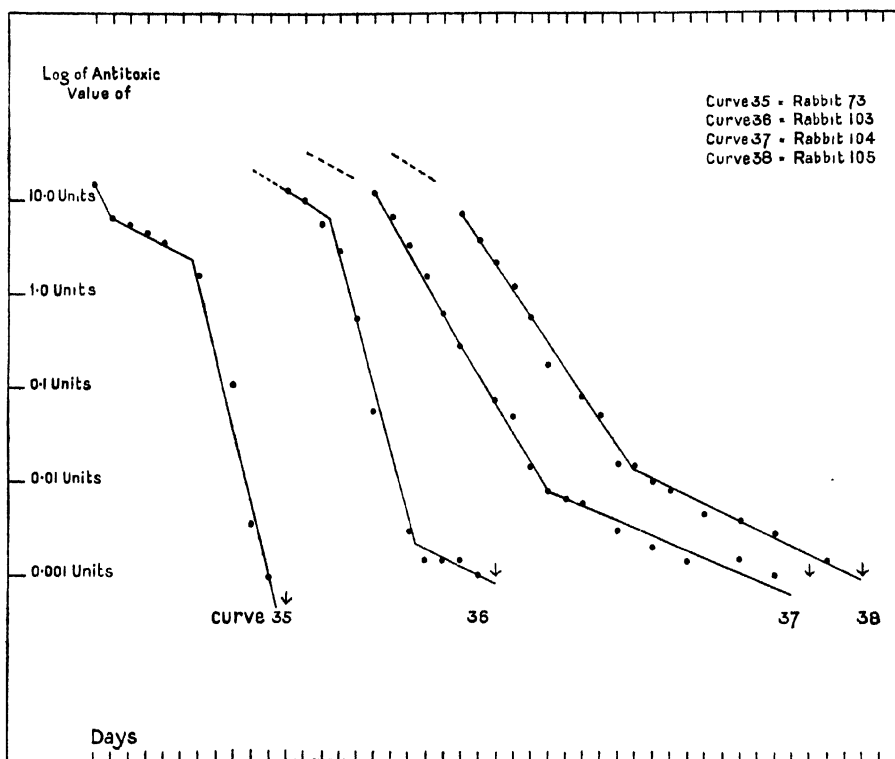


Chart IX.

tables show that the immunity reaction of rabbits to horse serum, in so far as elimination of the antitoxin carrying pseudo-globulin is concerned, is the same whether the rabbit is injected with whole serum or with purified pseudo-globulin.

All results so far recorded have dealt with rabbits injected intravenously. Two normal rabbits 55 and 63 were injected subcutaneously, one with the usual amount of horse serum (0.5 c.c. containing 750 units of antitoxin) and the other with ten times that amount. The results are recorded in Table XVIII and Chart XI. Owing to the slow rate of absorption after subcutaneous

Table XVI.

Showing the antitoxic content of three normal rabbits from the same litter at different intervals of time after the intravenous injection of different quantities of diphtheria antitoxin contained in purified horse pseudo-globulin.

Rabbit	106	107	108
Weight	990	880	920
Age	11 wks.	11 wks.	11 wks.
Units of antitoxin injected	75	750	7500
Volume	0.05 c.c.	0.5 c.c.	5.0 c.c.
Time interval			
15 minutes	1.67	16.0	130.0
1 day	0.58	6.5	55.0
2 days	0.45	4.5	37.0
3 "	0.3	3.5	30.0
4 "	0.18	2.75	22.0
5 "	0.14	2.25	17.5
6 "	0.11	0.58	12.0
7 "	0.045	0.1	9.0
8 "	0.02	0.02	4.7
9 "	—	—	—
10 "	<0.001	0.004	0.8
11 "	—	—	0.18
12 "	—	0.0015	0.05
13 "	—	<0.001	0.015
14 "	—	—	0.006
15 "	—	—	0.003
16 "	—	—	—
17 "	—	—	0.001
18 "	—	—	—
19 "	—	—	<0.001

Table XVII.

Showing the percentage daily loss in antitoxic value of the blood of three normal rabbits from the same litter at different intervals of time after the intravenous injection of different quantities of diphtheria antitoxin contained in purified horse pseudo-globulin.

Time interval	Rabbit 106		Rabbit 107		Rabbit 108	
0-1 day	65.2	Phase A	59.4	Phase A	57.7	Phase A
1-2 days	22.4	Phase B Average 27.8	30.7	Phase B	32.7	Phase B Average 25.8
2-3 "	33.3		22.2	Average	18.9	
3-4 "	40.0		21.4	23.1	26.7	
4-5 "	22.2		18.2	Phase C Average 66.2	20.4	
5-6 "	21.4	Phase C Av. 57.3	74.2		31.4	Phase C Average 59.8
6-7 "	59.1		82.7		25.0	
7-8 "	55.5		80.0		47.7	
8-9 "	—	—	—	—	—	—
9-10 "	—		55.3		58.7	
10-11 "	—		—		77.5	
11-12 "	—		38.8		72.2	
12-13 "	—	—	—	—	70.0	Phase C Average 59.8
13-14 "	—		—		60.0	
14-15 "	—		—		50.0	
15-16 "	—		—		—	
16-17 "	—	—	—	—	42.3	—
17-18 "	—		—		—	
18-19 "	—		—		—	

injection, all appearance of Phase A is obliterated and a considerable portion of Phase B is also masked. The chief interest of the experiment lies in the comparison between the curves of antitoxic content of rabbits injected subcutaneously, and those of rabbits injected intravenously. On Chart XI the standard curve of total antitoxic content has been reproduced from Chart V. Curve 43, representing rabbit 63, has been scaled down to one-tenth for comparison. It will be seen that during the first two days the antitoxic content of the rabbit injected subcutaneously is markedly below the average content of rabbits injected intravenously. After that time there is no great difference. For a period of three days the Phase B section of the curve follows closely

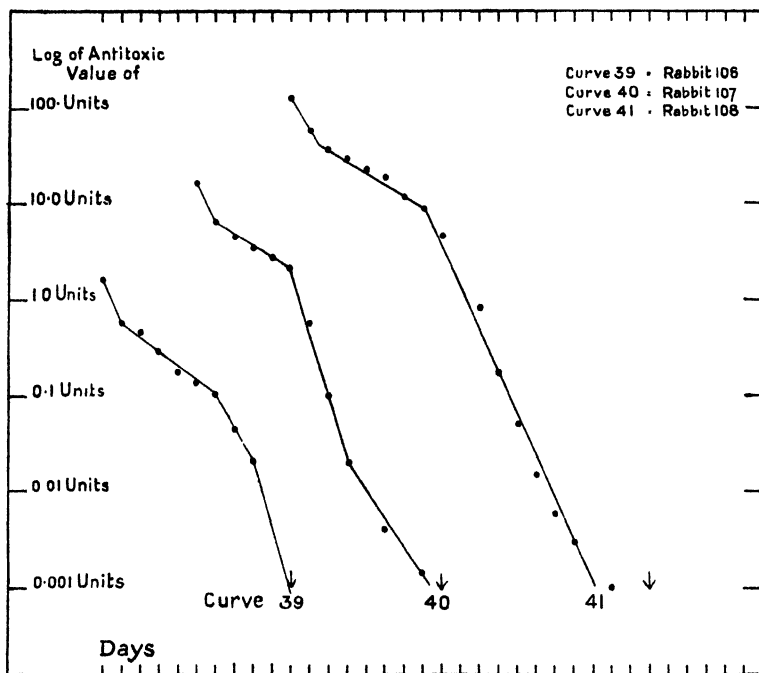


Chart X.

that of the standard curve for intravenous injection; the Phase C section follows closely that of curve 19 representing rabbit 41. The time of appearance and steepness of slope of Phase C show that the production of precipitin follows equally well the intravenous or the subcutaneous injection of horse serum. The relative antitoxic content after intravenous or subcutaneous injection is also seen in Table XVIII by comparing the actual values of rabbit 63 injected subcutaneously with those given for rabbit 72. As already recorded in Table XII, rabbit 72 was injected with 5 c.c. of horse serum; because only one-tenth of the serum was antitoxic horse serum of the same unit value as that injected into rabbit 63, the observed values of rabbit 72 have been multiplied by ten. The differences of weight of the two rabbits is

Table XVIII.

Showing the antitoxic content of the blood of two rabbits injected subcutaneously with antitoxic horse serum.

Rabbit	55	63	72
Weight	1930	1790	1590
Volume of serum injected	0.5 c.c.	5.0 c.c.	5.0 c.c.
Number of units injected	750	7500	750
Method of injection	Subcutaneously		Intravenously
Time interval	Antitoxic value in units per c.c.		10 times antitoxic value in units per c.c.
1 hour	—	0.25	—
2 "	—	0.35	75.0
3 "	—	1.3	—
4 "	—	2.25	70.0
5 "	—	3.25	—
6 "	—	4.25	65.0]
7 "	—	—	—
8 "	—	7.0	—
1 day	2.75	19.0	45.0
2 "	3.5	28.0	32.5
3 "	3.5	28.0	27.5
4 "	2.75	19.0	22.5
5 "	2.75	—	13.2
6 "	—	11.0	7.0
7 "	0.70	6.0	2.5
8 "	0.0008	2.75	1.4
9 "	0.00015	1.3	0.8
10 "	<0.0005	0.45	0.3
11 "	—	0.022	0.1
12 "	—	—	0.06
13 "	—	<0.0005	—

not sufficient to affect the following comparison. Two hours after injection, the antitoxic value per c.c. of blood of a rabbit injected intravenously was 200 times that of a rabbit injected subcutaneously with the same dose; four hours after injection the value was 30 times, six hours after, 15 times, 24 hours after injection twice, two days after injection 15 per cent. above, and not until the third day did the values coincide. Although many workers have called attention to the advantage of intravenous injection of antitoxin over subcutaneous injection, the importance of this point does not yet seem to be fully realised. Curve 43A on Chart XI shows the rate of absorption of antitoxin during the first eight hours after injection. Very little antitoxin is absorbed during the first two hours and then about 1 per cent. of the total injected is absorbed per hour. The highest concentration in the blood, two and three days after injection, represents only one-third of the total antitoxin injected.

It is convenient at this stage to consider the variations in response of the normal rabbits injected with horse serum.

Table XIX has been prepared in order to summarise one aspect of the varied responses of different rabbits to horse serum. The rabbits have been arranged in order of weight which, to a certain extent, indicates age, and to

Duration of Passive Immunity

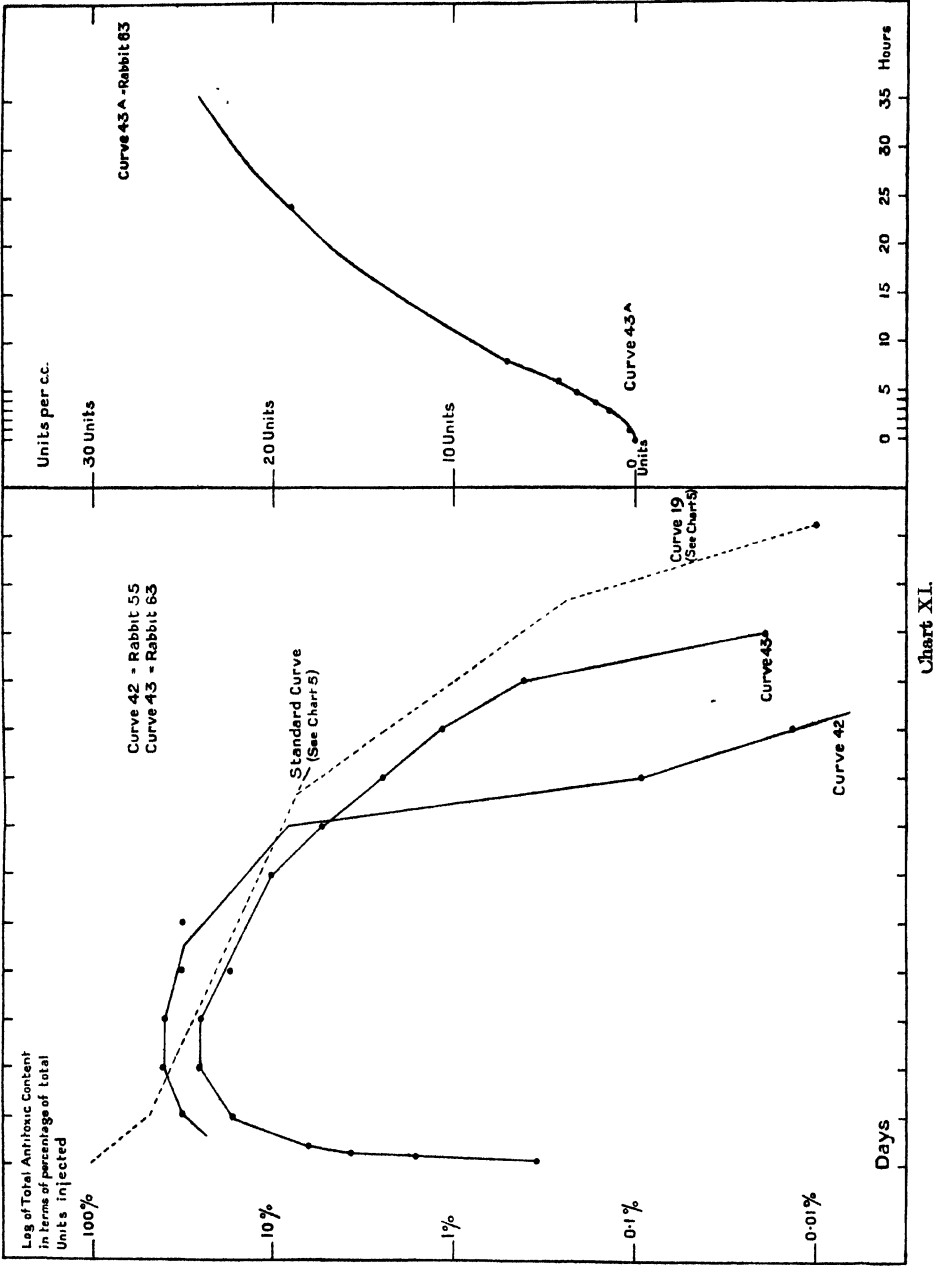


Table XIX.

Showing the number of days taken for the antitoxic content of all normal rabbits recorded in preceding tables to fall below various stated fractions of the total amounts injected.

Rabbit	Table	Weight	1/10	1/100	1/1000	1/10,000	1/100,000
84	10	400	7	11	12	13-14	—
76	8	510	7	13-15	19	26	—
86	8	680	6-7	12	19	27-30	—
79	8	740	6-7	9	10	12	—
107	16	880	6	7	9-10	12	—
94	10	910	6	6	7	9	—
108	16	920	6	9-10	12	14	17
106	16	990	5	9-10	9-10	—	—
93	8	1020	6	8-9	12	16	—
75	12	1190	6-7	9	10	11-12	—
73	14	1390	5	7-8	9	10	—
71	12	1470	6	10	12	—	—
72	12	1590	6	9	12	c. 14	—
105	14	1620	6	10	12-13	17	28-29
103	14	1640	7	10	11	12-13	17
41	1	1810	7	11	12-13	14-16	—
50	1	1870	8	10	—	—	—
49	1	1930	7	10	12	12	—
104	14	1930	7	10	12-13	16	29
42	5	2040	8-9	11	13	21-23	—
61	5	2040	5	7	8	8	—
74	12	2380	6-7	8-9	9	10	12
110	10	2810	5	6	6	7	—
109	10	2840	6	6	7	8	—
83	10	3240	6-7	6-7	7	8	—

each rabbit is marked the number of days taken for the antitoxic content to fall below one-tenth, one-hundredth, one-thousandth, etc. of the original content. It will be seen that 25 normal rabbits injected with antitoxic horse serum intravenously lost 90 per cent. of the total antitoxin in from 5 to 9 days, while 19 of the 25 rabbits lost this amount by the sixth or seventh day; no marked connection can be seen between variations from the average and the weight of the animal. The next column of the table shows that various rabbits have taken from 6 to 13 or 15 days to lose 99 per cent. of the antitoxin injected. The majority of the rabbits have taken from 10 to 11 days to lose this quantity. The only rabbits to take more than 11 days are quite small, although four out of eight of those weighing less than one kilo. took less than ten days. The times taken to lose 99.9 per cent. of the antitoxin injected, *i.e.* for the antitoxic content to fall below one-thousandth of the original value give more consistent results in accordance with weight or age. The average time taken is from 10 to 13 days; of the rabbits weighing less than one kilo. four took 10 days or less, two took 12 days and two 19 days; of the rabbits weighing between one and two kilos. one took under 10 days, nine took from 10 to 13 days, and none longer; five out of six of those weighing over two kilos. took less than 10 days. The time taken to fall to one ten-thousandth of the original value was more varied; the average time would appear to be about 12 to 16 days but this time varied from seven days in a heavy rabbit to between 27 to 30 days in a light rabbit. The rabbit giving the most abnormal results was rabbit 94;

although under one kilo. at the time of injection and only seven weeks old it responded so markedly to horse serum that all detectable antitoxin was lost in nine days.

There is already sufficient evidence to show that older rabbits tend to exhibit an increased capacity for response to an injection of horse serum, which may be regarded as an acquired active immunity. It would be of interest to determine how this active immunity can be acquired.

It is possible that active immunity may have been acquired by feeding, but if immunity may be acquired with such ease then it would appear possible for rabbits to acquire an active immunity before birth or to have absorbed the antigen. Tables XX and XXI and curves 44 to 46 on Chart XII record the results obtained from three rabbits that may have some bearing on this point. Rabbits 80 and 92 were both born of the same litter from a mother highly immune to horse serum and injected several times during pregnancy. Rabbit 80 injected with horse serum when only five weeks old eliminated serum more rapidly than was usual for young rabbits, but rabbit 92 injected when ten weeks old eliminated even more rapidly. If the rapid elimination of antitoxic horse serum was due to passive immunity inherited from the mother, the loss due to Phase A would have been greatly increased by elimination due to circulating precipitin and the older rabbit should have appeared less immune.

It is possible that rabbit 80 possessed circulating precipitin at the time of injection; a first day loss of 55.5 per cent. recorded in Table XXI was succeeded by a second day loss of 54.2 per cent., but as reference to Table XX shows

Table XX.

Showing the antitoxic content of three rabbits at different intervals of time after the intravenous injection of 750 units of diphtheria antitoxin contained in 0.5 c.c. of horse serum.

Rabbit	80	92	85
Weight	510	1110	2380
Age	5 wks.	10 wks.	—
Previous history	Rabbits of the same litter born from a mother highly immune to horse serum		Fed with horse serum 3 weeks previously
Time interval			
15 minutes	27.0	15.0	8.0
1 day	12.0	7.5	4.0
2 days	5.5	5.25	—
3 "	—	—	2.5
4 "	5.5	3.25	2.25
5 "	3.0	1.25	0.7
6 "	—	0.11	0.02
7 "	0.22	0.015	<0.001
8 "	0.14	0.002	—
9 "	0.08	0.001	—
10 "	0.018	—	—
11 "	0.008	—	—
12 "	<0.0025	<0.0005	—
13 "	—	—	—
14 "	0.0014	—	—
15 "	<0.0005	—	—

Table XXI.

Showing the percentage daily loss in the antitoxic value of the blood of three rabbits at different intervals of time after the intravenous injection of 750 units of diphtheria antitoxin contained in 0.5 c.c. of unconcentrated horse serum.

Time interval	Rabbit 80		Rabbit 92		Rabbit 85	
0-1 day	55.5	Phase A	50.0	Phase A	50.0	Phase A
1-2 days	(54.2)?		30.0	Phase B	—	Phase B
2-3 "	—	Phase B	—	Average	21.0	Average
3-4 "	(22.9)	Av. 22.9	21.3	25.6	10.0	15.5
4-5 "	45.4		61.5		68.9	Phase C
5-6 "	—		91.2	Phase C	97.1	Av. 83.0
6-7 "	72.9		86.4	Average		
7-8 "	36.4		86.7	75.1		
8-9 "	42.9	Phase C	50.0			
9-10 "	77.5	Average 53.0				
10-11 "	55.5					
11-12 "	68.7					
12-13 "	—					
13-14 "	25.2					

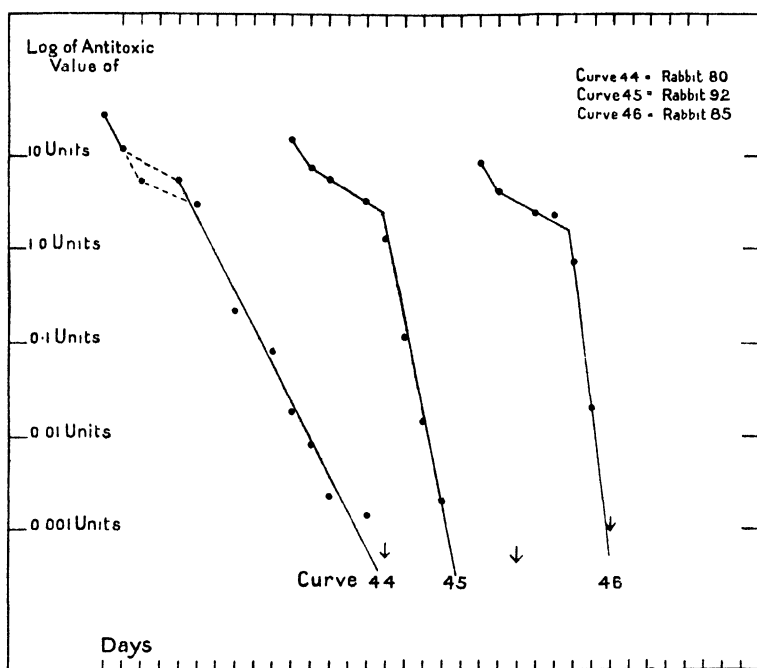


Chart XII.

that the antitoxic values recorded both for the second and for the fourth day was 5.5 units per c.c., it is obvious that one value recorded was an incorrect reading. If the second day reading is correct, then Phase A augmented by elimination due to circulating precipitin extended over two days, Phase B ends on the fifth day and the average rate of loss calculated from the readings of 5.5 units on the second day and 3.0 on the fifth day was 26.2 per cent. On the other hand if the fourth day reading is correct then passive immunity

inherited from the mother was already lost when the rabbit was injected at the age of five weeks, and Phase B ended on the fourth day and showed an average loss of 22·9 per cent. calculated from the first day reading of 12·0 unit and the fourth day reading of 5·5 unit.

Table XXII shows the number of days taken for the total antitoxin content of these rabbits to fall below one-tenth, one-hundredth, one-thousandth, and one-ten-thousandth of the total amount injected. This table read in conjunction with Table XIX compiled in a similar manner for normal rabbits, emphasises the fact that rabbit 80 eliminated antitoxin more quickly than the majority of rabbits weighing less than 1 kilo., while rabbit 92 (weighing only 1100 grams) exhibited a greater rate of loss than any of the eleven normal rabbits weighing between one and two kilos.

Table XXII.

Showing the number of days taken for the antitoxic content of the rabbits recorded in Table XX to fall below various stated fractions of the total amount injected.

Rabbit	Weight	1/10	1/100	1/1000	1/10,000
80	510	6-7	6-7	10	12
92	1110	5	6	7	9
85	2380	5	6	7	7

Rabbit 85 had been fed with horse serum three weeks before the injection and showed a high degree of immunity. This may indicate the possibility that active immunisation to horse serum readily arises from feeding or simply that older rabbits may, for some reason not understood, be highly immune.

That some degree of sensitiveness (*i.e.* immunity) to horse serum may be acquired by rabbits in some accidental way may not appear an unreasonable hypothesis when we consider the remarkable instances of sensitiveness of different human beings to different proteins.

SUMMARY OF PART II.

1. Normal rabbits injected intravenously with diphtheria antitoxin obtained from a horse vary considerably both in regard to the duration of Phase B and the duration and the intensity of Phase C.

2. Phase C, the phase of accelerated loss due to formation of precipitin appears sooner and is more pronounced in older rabbits.

3. The early appearance and more rapid formation of antibody (precipitin) in certain rabbits is indicative of naturally acquired immunity.

4. The highest concentration in the blood of rabbits injected subcutaneously with antitoxic horse serum is seen after 2 to 3 days, when it reaches approximately the same value as that of rabbits injected intravenously, and represents only one-third of the total antitoxin injected. Two hours after subcutaneous injection the antitoxic content is only one two-hundredth of that of a rabbit injected intravenously.

DURATION OF PASSIVE IMMUNITY.

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(Wellcome Physiological Research Laboratories, Beckenham, Kent.)

PART III.

(With Charts XIII-XVI.)

PARTS I and II of this paper have dealt with the three phases in the course of elimination of passive immunity in rabbits injected with diphtheria antitoxin obtained from a horse. The present section deals with other normal animals injected with horse serum, normal rabbits injected with serum from animals other than the horse, and with animals injected with homologous antitoxin.

A number of normal guinea-pigs were injected subcutaneously with 5 c.c. of horse serum containing 2500 units of diphtheria antitoxin, and blood was withdrawn from the heart on successive days after the injection. The results of antitoxin titration of the samples of serum so obtained are given in Table XXIII. Because each value recorded represents a different guinea-pig, there

TABLE XXIII.

Showing the antitoxic value of the serum of a number of guinea-pigs bled at different intervals of time after the subcutaneous injection of 5 c.c. unconcentrated horse serum containing 2500 units of diphtheria antitoxin.

Guinea-pig	Days after injection	Weight in grams	Units of antitoxin per c.c.
A	1	280	30
B	2	355	25
C	2	300	35
D	3	260	40
F	5	300	25
H	6	280	12
K	7	325	7
L	8	280	7
N	9	310	4
O	10	260	0.3
P	13	285	0.03
R	14	250	0.03
S	15	300	0.005
T	16	305	0.02
W	22	285	<0.001

is considerable variation among the values obtained, but the figures are sufficient to show that the course of elimination of antitoxic horse serum from the guinea-pig closely resembles that of similar serum from the rabbit. A guinea-pig tested 22 days after injection, contained in its circulation less than 1/100,000 of the total antitoxin injected. On comparing the general trend of

the figures in Table XXIII with those in Table XVIII and Chart XI in Part II it will be seen that after subcutaneous injection of both rabbits and guinea-pigs with antitoxic horse serum the maximum concentration is obtained in the blood two or three days after the injection, absorption appears complete by the third or fourth day and the curve of loss then follows the normal course of Phase B. Precipitin formation represented by Phase C commences slightly later in guinea-pigs; guinea-pig *O* on the tenth day contained only one-tenth of the antitoxin seen in guinea-pig *N* on the ninth day, showing that in guinea-pig *O* Phase C had commenced by the tenth day. This result is in line with the generally accepted facts of anaphylaxis in guinea-pigs. One guinea-pig, *T*, showed an exceptionally high antitoxic value on the sixteenth day after injection.

TABLE XXIV.

Showing the antitoxic content of rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in goat serum.

Rabbit ...	88	118	45
Weight in grams	1700	1340	1760
Immunity ..	Normal	Normal	Horse sensitive
Volume injected	5.0 c.c.	5.0 c.c.	5.0 c.c.
Total units ...	125	125	50
Antitoxic value in units per c.c.			
Time interval			
15 minutes	—	2.5	0.5
1 day	0.9	0.9	0.3
2 days	0.55	0.55	0.25
3 "	—	—	0.18
4 "	0.35	0.18	0.14
5 "	0.08	0.08	0.12
6 "	<0.0005	0.006	—
7 "	—	<0.001	0.015
8 "	—	—	0.001
9 "	—	—	<0.0005

TABLE XXV.

Showing the percentage daily loss in antitoxic value of the blood of rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in goat serum.

Time interval	Rabbit 88	Rabbit 118	Rabbit 45
0-1 day	— Phase A	64.0 Phase A	40.0 Phase A
1-2 days	38.9 Phase B	38.9 Phase B	16.7 Phase B
2-3 "	— Average	— Average	28.0 Average
3-4 "	20.2 29.5	42.8 40.8	22.2 20.3
4-5 "	77.1 Phase C	55.5 Phase C	14.3
5-6 "	99.3 Average	92.5 Average	— Phase C
6-7 "	88.2	74.0	64.6 Average
7-8 "	—	—	93.3 78.9

Three rabbits were injected intravenously with diphtheria antitoxin obtained from a goat. The results are recorded in Tables XXIV and XXV and Chart XIII. The outstanding features of each of the three curves 47, 48 and 49 is the early appearance of Phase C and the shortness of the period during which antitoxin was present. Rabbit 45, already sensitised to horse serum, did not

eliminate goat serum with the rapidity with which similarly sensitised rabbits eliminate horse serum, and showed less, rather than more, activity of response to goat serum than did the normal rabbits 88 and 118. The curve for rabbit 45 exhibits the typical three phases of elimination; the absence of a 15-minute reading for rabbit 88 and the early appearance of Phase C obscure the actual course of Phase B in Curve 47. Curve 48 is strongly suggestive of Curve 14 on Chart IV in Part II representing the course of elimination of horse serum from a rabbit injected only 11 days after a previous injection at a time when excess precipitin would still be present in the circulation. This suggestion of existence of circulating precipitin in one rabbit, and the early appearance and

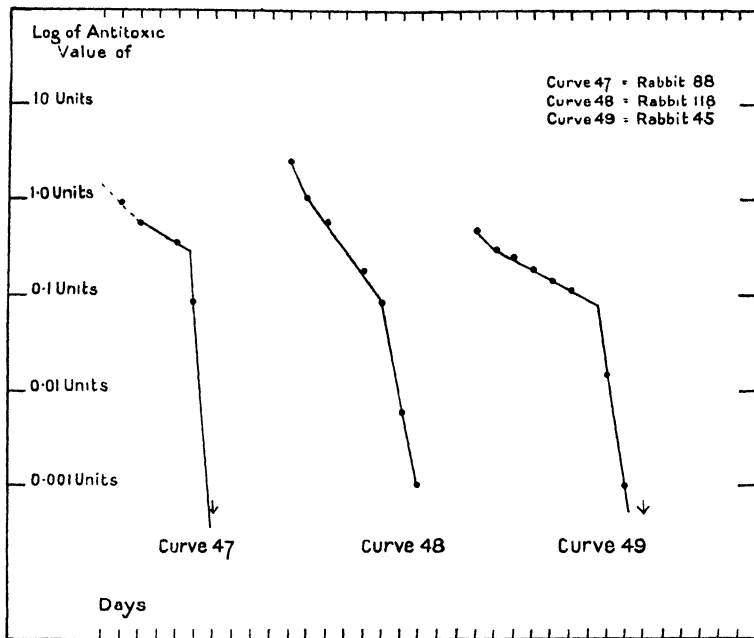


Chart XIII.

short duration of Phase C in all three rabbits shows that rabbits are more responsive to goat serum than to horse serum. Table XXVIII, showing the time taken for antitoxic values to fall below certain levels, also shows, when compared with Table XIX, that goat serum is eliminated more rapidly than horse serum. It must be pointed out that if immunity to animal protein is specifically acquired, the evidence suggests that the particular rabbits tested had more opportunity of acquiring immunity to goat serum than similar rabbits to horse serum. Unfortunately, no record was kept as to which of these rabbits had been acquired from outside sources; rabbits 45 and 88 were known to have been tended by assistants closely associated with the care of goats.

Tables XXVI and XXVII and Curves 50 to 55 on Chart XIV record the results of injecting rabbits intravenously with antitoxic serum obtained from men, guinea-pigs and cows. The rate of loss of antitoxin from these rabbits

and also from those recorded in Table XXIV are summarised in Table XXVIII. The antitoxin injected had been actively produced. Rabbit 91 is of special interest, the antitoxic content 15 minutes after injection was less than half the calculated value, and the loss during the next 24 hours was 80·6 per cent. These facts are strongly suggestive of the presence of circulating precipitin. The general results obtained indicate that the rabbits used in these experiments were particularly responsive to the serum for goat, guinea-pigs and men, less so to serum from horses and still less to serum from cows. These rabbits

TABLE XXVI.

Showing the antitoxic content of rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in cow, guinea-pig and human serum.

Rabbit	87	47	91	117	78	89
Weight	1580	1420	1110	1390	—	1190
Immunity	Normal	Horse sensitive	Normal	Normal	Normal	Normal
Serum injected	Cow	Cow	Human	Human	Guinea-pig	Guinea-pig
Volume in c.c.	5·0	5·0	5·0	5·0	10·0	5·0
Total units	750	750	22·5	10	200	100

Time interval	Antitoxic value in units per c.c.					
	87	47	91	117	78	89
15 minutes	8·5	11·5	0·17	0·11	2·75	1·62
1 day	5·25	7·5	0·033	0·055	1·75	0·9
2 days	3·75	5·75	0·025	0·045	1·5	0·7
3 "	—	4·75	—	0·035	—	—
4 "	2·25	3·5	0·01 -0·04	0·03	0·9	0·55
5 "	0·9	2·75	0·01 -0·02	—	—	0·33
6 "	0·05	—	0·007	0·018	—	0·04
7 "	0·015	0·55	0·002-4	0·008	0·3	0·005
8 "	0·004	0·17	0·002	0·0045	—	<0·001
9 "	0·002	0·02	?0·0008	0·0015	—	—
10 "	—	0·01	—	0·0015	0·022	—
11 "	—	0·007	—	<0·001	Died	—
12 "	<0·0005	0·004	?0·0008	—	—	—
13 "	—	—	—	—	—	—
14 "	—	0·002	? <0·0005	—	—	—
15 "	—	0·002	—	—	—	—
16 "	—	0·0015	—	—	—	—
17 "	—	0·0015	—	—	—	—
18 "	—	0·0015	—	—	—	—
19 "	—	0·0015	—	—	—	—
20 "	—	—	—	—	—	—
21 "	—	0·0007	—	—	—	—
22 "	—	<0·0005	—	—	—	—

were in relatively close contact with goats, guinea-pigs and men, but not in direct contact with horses, while their connection with cows was even more remote. Considerably more work must be done before any positive proof can be obtained as to whether rabbits can acquire immunity to protein of other animals, but the facts at present known are sufficiently suggestive to warrant the mention of such a possibility.

TABLE XXVII.

Showing the percentage daily loss in antitoxic value of the blood of rabbits at different intervals of time after the intravenous injection of diphtheria antitoxin contained in cow, human and guinea-pig serum.

Time interval in days	Rabbit 87		Rabbit 47		Rabbit 91		Rabbit 117		Rabbit 78		Rabbit 89	
0-1	38.2	Phase A	34.8	Phase A	80.6	Phase A	50.0	Phase A	36.4	Phase A	44.4	Phase A
1-2	28.5	Phase B	23.3	Phase B Average	24.2	Phase B Average	18.2	Phase B Average	14.3	Phase B Average	22.2	Phase B Average
2-3	—	Average	17.4		—		22.2		—		—	
3-4	22.5	25.5	26.3		—		14.3		22.5		11.4	
4-5	60.0	—	21.4	Phase C Average	22.1	Phase C Average	25.7	Phase C Average	19.3	Phase C Average	40.0	Phase C Average
5-6	94.4	Phase C	—		27.2		22.5		—		87.8	
6-7	70.0	Average	55.3		42.7		55.5		30.6		87.5	
7-8	70.0	70.0	69.1	Phase D Average	—	Phase D Average	43.7	Phase D Average	—	Phase D Average	—	Phase D Average
8-9	55.5	—	88.2		51.5		66.7		—		—	
9-10	—	—	50.0		55.9		(49.2)		58.2		—	
10-11	—	—	30.0	Phase E Average	—	Phase E Average	—	Phase E Average	—	Phase E Average	—	Phase E Average
11-12	—	—	42.8		—		—		—		—	
12-13	—	—	—		—		—		—		—	
13-14	—	—	29.3	Phase F Average	—	Phase F Average	—	Phase F Average	—	Phase F Average	—	Phase F Average
14-15	—	—	(20.6)		—		—		—		—	
15-16	—	—	(25.0)		—		—		—		—	
16-17	—	—	(13.4)	Phase G Average	—	Phase G Average	—	Phase G Average	—	Phase G Average	—	Phase G Average
17-18	—	—	(9.1)		—		—		—		—	
18-19	—	—	(6.9)		—		—		—		—	

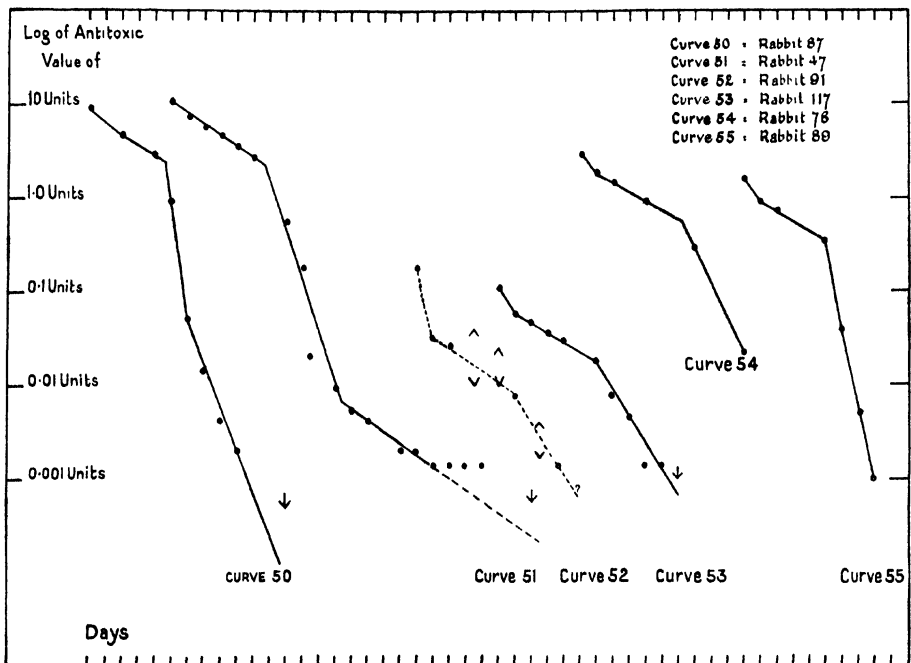


Chart XIV.

TABLE XXVIII.

Showing the number of days taken for the antitoxin content of the rabbits recorded in Tables XXIV and XXVI to fall below various stated fractions of the total amount injected.

Rabbit	Serum injected	Weight	1/10	1/100	1/1000	1/10,000
88	Goat	1700	5	6	6	—
118	"	1340	4	6	7	—
45	"	1760	6-7	7	8	—
87	Cow	1560	6	6	8	10-12
47	"	1420	6-7	9	10	20-21
91	Human	1110	6	9	—	—
117	"	1390	7	12	—	—
78	Guinea-pig	?	8	9-10	—	—
89	"	1190	6	7	8	—

The rate of loss of passive immunity produced by injection of homologous antitoxin may now be considered. A rabbit was injected intravenously with antitoxin actively produced in another rabbit and three horses were injected with antitoxin contained in horse serum freed from euglobulin and from albumen. Details relating to these animals are given in Tables XXIX and XXX and Curves 56 to 59 in Chart XV. Two curves are given for rabbit 77; in order to show in detail the course of antitoxic loss in such a form as to be comparable with preceding curves, Curve 56 A has been drawn on a larger scale (showing daily readings) than Curve 56 showing weekly readings to compare with 57, 58 and 59. The table of percentage loss and curve 56 A show that the rate of loss is not quite uniform. Throughout the whole period except for the first reading, involving, as it does, Phase A, the average weekly loss of rabbit serum injected into a rabbit is 57.2 per cent., equivalent to a daily loss of 11.1 per cent. of each day's content. For the first 17 days a greater loss takes place, 69.2 per cent. per week representing 15.5 per cent. per day, or taking the actual readings of 0.14 of a unit on the 17th day and 1.87 of a unit on the first day and abstracting the 16th root we get the daily loss as 15.0 per cent. The reason for this change in rate of loss after 17 days is not apparent. The loss during the first 24 hours representing Phase A was 42.4 per cent.; the loss during the next 24 hours was 26.7 per cent. compared with 9.7 per cent. per day for the next two days and 14.5 per cent. for the following three days. The high figure for the second day loss is suggestive of an extended Phase A due to the relatively large volumes (10 c.c.) of serum injected.

The horses were tested at weekly intervals only. Curve 57 for horse 980 shows a marked drop representing Phase A and then a steady loss until the animal is again injected at the sixth week. This injection was followed again by a Phase A loss, and then a steady fall averaging 17.5 per cent. per week. Individual readings for weekly differences vary considerably from this average, but, with so many points determined upon a single curve, it was unnecessary to titrate accurately each sample taken. From this weekly loss and the calculated loss during the first seven days it would appear that Phase A consisted of approximately a loss of 50 per cent. The curve of loss of antitoxin in horse 981

TABLE XXIX.

Showing the antitoxic content of one normal rabbit and three normal horses at different intervals of time after the intravenous injection of diphtheria antitoxin contained in homologous serum.

Animal		Rabbit 77	Horse 980	Horse 981	Horse 982
Injection	Rabbit serum	Purified globulin from horse serum			
volume	10.0 c.c.	100 c.c.			
Units	145	100,000			
Antitoxic value in units per c.c.					
Time interval					
15 minutes	3.25	4.0	3.25	3.25	
1 day	1.87	—	—	—	
2 days	1.37	—	—	—	
4 "	1.12	—	—	—	
7 "	0.7	1.75	2.5	2.25	
1½ weeks	0.5	—	—	—	
2 "	0.2	1.25	1.5	1.5	
2½ "	0.14	—	—	—	
3 "	0.11	1.25	1-1.5	1.25	
3½ "	0.07	—	—	—	
4 "	0.04	1.0	1-1.25	1.0	
4½ "	0.35	—	—	—	
5 "	0.02	0.8	.75-1.0	.8-1.0	
5½ "	0.017	—	—	—	
6 "	0.009	4.5*	.6	.6	
6½ "	0.007	—	—	—	
7 "	0.0045	2.5	.6	.5	
7½ "	0.003	—	—	—	
8 "	0.0035	2.0	.4	.5	
8½ "	0.001	—	—	—	
9 "	0.0015	1.75	.25	.4	
9½ "	0.0015	—	—	—	
10 "	0.0007	1.25	.15	.4	
10½ "	0.0005	—	—	—	
11 "	<0.0005	1.25	.16	.25	
12 "	—	1.0	.142	.2	
13 "	—	.8	.11	.16	
14 "	—	.8	.125	.14	
15 "	—	.6	.09	.16	
16 "	—	.5	.067	.14	
17 "	—	.4	.05	.125	
18 "	—	.33	.04	.125	
19 "	—	.25	.03	.11	
20 "	—	.25	.025	.1	
21 "	—	.2	.022	.07	
22 "	—	.17	.018	.07	
23 "	—	.14	.014	.07	
24 "	—	.12	.011	.05	
25 "	—	.1	.008	.04	
26 "	—	.08	.007	.04	
27 "	—	.06	.0045	.044	
28 "	—	.045	.004	.035	
29 "	—	.045	—	.025	
30 "	—	.035	.002-4	.025	
31 "	—	.025	.002	.025	
32 "	—	.025	.001-2	.025	
33 "	—	.018	.001-2	.025	
34 "	—	—	.001-2	.02	
35 "	—	.016	.001-2	.02	
36 "	—	—	.001	.012	
37 "	—	.01	.001	.025	
38 "	—	—	.001	.033	
39 "	—	.006	—	.033	
40 "	—	.006	—	.025	
41 "	—	.006	—	.033	
42 "	—	.004	—	.025	
43 "	—	.004	—	.02	
44 "	—	.003	—	.025	
45 "	—	.003	—	.067	
46 "	—	.002	—	.067	
47 "	—	.0015	—	.25	
48 "	—	—	—	.33	
49 "	—	.0015	—	1.25	
50 "	—	—	—	1.5	

TABLE XXX.

Showing the percentage weekly loss in antitoxic value of the blood of one normal rabbit and two normal horses at different intervals of time, after the intravenous injection of diphtheria antitoxin contained in homologous serum.

Time interval	Rabbit 77	Horse 980	Horse 981
0 -1 week	78.5	56.2	23.1
$\frac{1}{2}$ -1 $\frac{1}{2}$ weeks	61.0		
1 -2 "	71.4	28.5	40.0
1 $\frac{1}{2}$ -2 $\frac{1}{2}$ "	72.0		
2 -3 "	45.0	0.0	(0.33.3)
2 $\frac{1}{2}$ -3 $\frac{1}{2}$ "	50.0		
3 -4 "	63.6	20.0	(8.7-18.4)
3 $\frac{1}{2}$ -4 $\frac{1}{2}$ "	50.0		
4 -5 "	50.0	20.0	(12.6-20.6)
4 $\frac{1}{2}$ -5 $\frac{1}{2}$ "	51.4		
5 -6 "	55.0	0.0	(20.0-40.0)
5 $\frac{1}{2}$ -6 $\frac{1}{2}$ "	58.8		
6 -7 "	50.0	44.4	0.0
6 $\frac{1}{2}$ -7 $\frac{1}{2}$ "	57.1		
7 -8 "	(22.2)	20.0	33.3
7 $\frac{1}{2}$ -8 $\frac{1}{2}$ "	(66.7)		
8 -9 "	(57.1)	12.5	37.5
8 $\frac{1}{2}$ -9 $\frac{1}{2}$ "	(+ 50.0)		
9 -10 "	53.3	28.6	0.0
9 $\frac{1}{2}$ -10 $\frac{1}{2}$ "	66.7		
10 -11 "	—	0.0	36.0
11 -12 "	—	20.0	11.3
12 -13 "	—	20.0	(22.5)
13 -14 "	—	0.0	(+ 12.7)
14 -15 "	—	25.0	25.5
15 -16 "	—	16.7	25.3
16 -17 "	—	20.0	20.0
17 -18 "	—	17.5	25.0
18 -19 "	—	24.2	16.7
19 -20 "	—	0.0	12.0
20 -21 "	—	20.0	18.2
21 -22 "	—	15.0	22.2
22 -23 "	—	17.6	21.4
23 -24 "	—	14.3	27.3
24 -25 "	—	16.7	12.5
25 -26 "	—	20.0	35.7
26 -27 "	—	25.0	11.1
27 -28 "	—	25.0	—
28 -29 "	—	0.0	—
29 -30 "	—	22.2	(20.5)
30 -31 "	—	28.5	—
31 -32 "	—	0.0	—
32 -33 "	—	28.0	—
33 -34 "	—	—	—
34 -35 "	—	(5.7)	—
35 -36 "	—	—	—
36 -37 "	—	(21.0)	—
37 -38 "	—	—	—
38 -39 "	—	(22.5)	—
39 -40 "	—	0.0	—
40 -41 "	—	0.0	—
41 -42 "	—	33.3	—
42 -43 "	—	0.0	—
43 -44 "	—	25.0	—
44 -45 "	—	0.0	—
45 -46 "	—	33.3	—
46 -47 "	—	25.0	—

Average 56.5 % Average 17.5 % Average 21.5 %

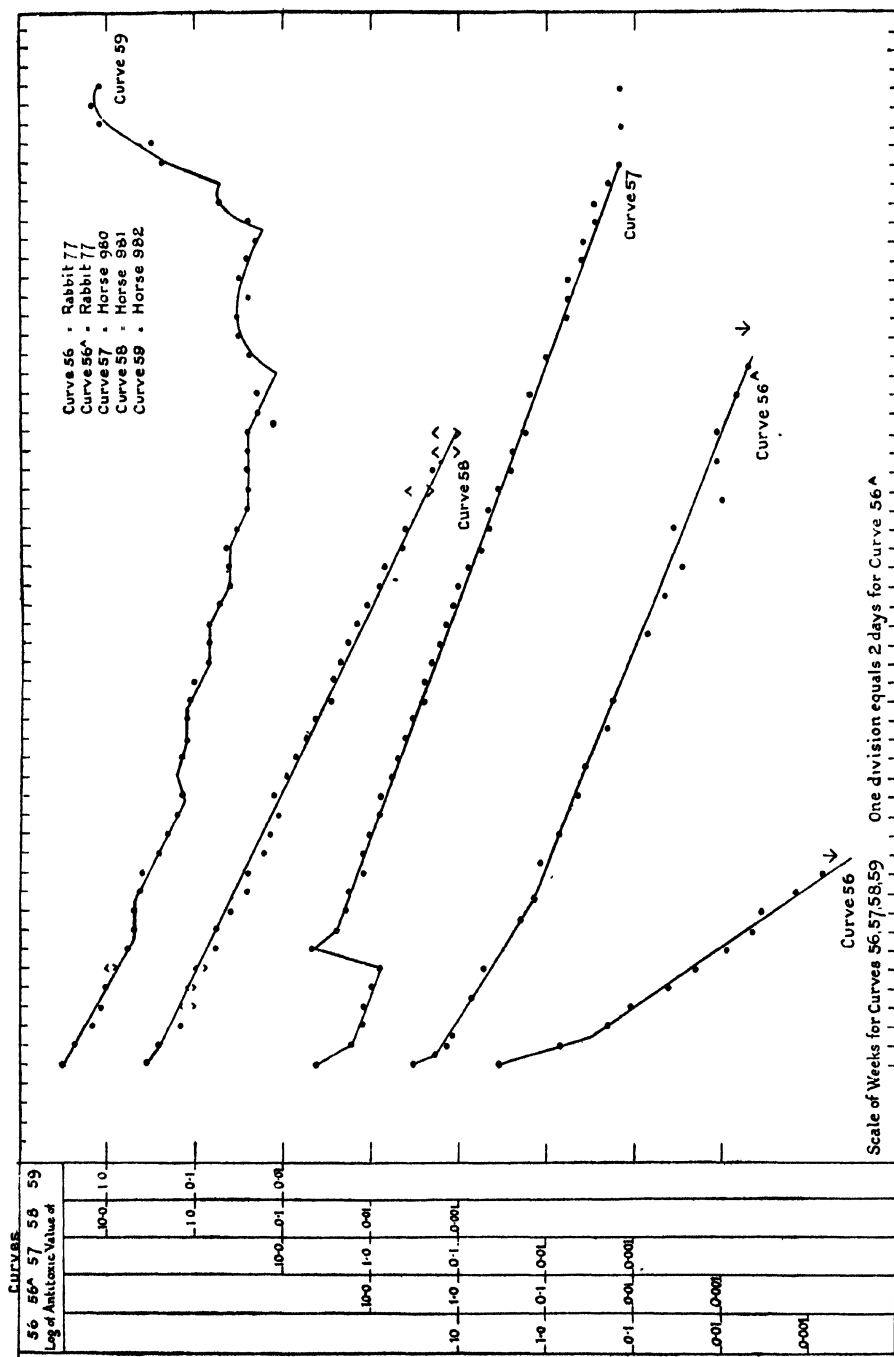


Chart XV.

again shows a straight line when the log of antitoxic content is plotted against time; this curve of loss is slightly steeper than that for horse 980, and represents a weekly loss of 21.5 per cent. Phase A is not evident in horse 981, but the high percentage loss from the first to the second week would indicate that the reading for the antitoxic content of the sample of blood, taken seven days after the injection, was too high. All antitoxin injected had disappeared about 35 weeks after injection. Horse 982, however, still showed a high antitoxic content over 40 weeks after injection and little or no loss occurred from the 29th to the 40th week and then a marked increase in antitoxic values was seen. The various readings of the antitoxic content of the weekly samples are very irregular and do not at first sight fall upon a recognisable curve, for the logs of the antitoxic content plotted against time, do not fall upon a straight line. It is, however, possible to draw, as we have done in Curve 59, a series of short sections parallel to Curve 58; these sections are linked with lines roughly parallel to the base line or showing a tendency to rise. Each such interrupted section represents an active immunity response. At the time of the injection of diphtheria antitoxin this horse already possessed a normal antitoxic value of 0.008 of a unit per c.c.; the other two horses, 980 and 981, both contained less than 0.0005 of a unit. Horse 982, already actively immune, could respond to any accidental stimulus by a rapid production of antitoxin, thus showing an irregular curve made up of constantly diminishing passive immunity and frequently varying active immunity. This horse affords a good illustration of the course of production of active immunity naturally acquired by an animal. At first the responses are small, and the rate of loss of apparent immunity is delayed only two weeks, but later the responses increase until eventually an increase in antitoxic content of one unit may occur within a week.

An examination of Curve 58, representing horse 981, also shows a marked tendency for a series of observed values to lie on one side of the straight line representing the average rate of loss of antitoxin. It is possible that these variations are of significance; it would be possible to draw a series of interrupted sections, *e.g.* 0-6, 7-13, 14-19 weeks, with small increases of active immunity between the sections.

The apparent delay between the 32nd and the 38th week before the antitoxic content fell below one-thousandth of a unit per c.c. may have been due to some small residual actively produced antitoxin; on the other hand, it must be stated that at the time these tests were made we were encountering some little difficulty in detecting small amounts of antitoxin. This detection of very small amounts of antitoxin depends upon the potency of the test toxin used and upon the relatively high content of toxin compared with toxoid. The minimal reacting dose (M.R.D.) of our test toxin at the commencement of our experiments was sufficiently small to enable one two-thousandth of antitoxin per c.c. to be detected with ease. Gradual deterioration of toxin into toxoid increased the M.R.D. in relation to L_R so that a reaction no longer appeared when tests were made at the $L_R/20,000$ level (see Glenny

and Allen, 1921, and Hartley and Hartley, 1922). In later experiments no attempt was made to titrate for less than one-thousandth unit per c.c. with this toxin, and for a short time, until another toxin was available, it was found difficult to ascertain definitely whether one-thousandth unit was present or not.

The majority of apparently normal horses possess some diphtheria antitoxin, in their blood, probably actively produced as a result of a succession of naturally acquired stimuli. The immunity responses to these stimuli gradually increase as may well be seen in the case of horse 982. It is possible, therefore, to regard horse 981 (and possibly horse 980) as in an earlier stage of actively acquired immunity to diphtheria, showing only very small responses to minute accidental stimuli. If this is so then the rate of loss of homologous serum in a horse must be represented by a line slightly steeper than Curve 58. If, however, the slight variations in the titration of values for horses 980 and 981 are of no significance and the apparent delay in reaching our zero line is due to difficulty in testing for small amounts of antitoxin, then Curves 57 and 58 must be taken as representing the true rate of loss of homologous serum in the horse. The slope of Curve 56 shows how much faster homologous serum may be eliminated from a rabbit than from horses.

TABLE XXXI.

Showing antitoxic value at different ages of three guinea-pigs of the same litter passively immune by maternal transmission.

Guinea-pig	Age	Antitoxic value in units per c.c.	Weight in grams	Weight × unit value
C	At birth	2.6	100	260
F	"	2.6	100	260
C	21 days old	0.46	270	124
C	24 "	0.44	300	132
D	24 "	0.54	255	138
C	43 "	0.11	440	48
D	43 "	0.12	360	43
F	43 "	0.12	375	45
C	61 "	0.04	530	21
F	68 "	<0.04	475	< 18

The remaining experiments recorded in this part were performed by one of us (A. T. G.) in conjunction with our colleague Dr H. J. Sudmersen in 1912 and 1913 and hitherto unpublished. Table XXXI shows the rate of loss of homologous serum in guinea-pigs. A litter of three guinea-pigs borne by an actively immune mother were bled within a few hours of birth, and their blood was found to be of the same antitoxic strength as that of their mother. One or other of the young was bled at different ages, until, when ten weeks old, no antitoxin could be detected. Antitoxic values were not titrated by the intradermic method and the least detectable strength depended upon the amount of blood available; with small quantities of blood obtainable from guinea-pigs it was not possible to detect less than one twenty-fifth unit per c.c.; with larger quantities obtainable from larger animals one-hundredth unit could be titrated. With growing guinea-pigs the antitoxic content is continually

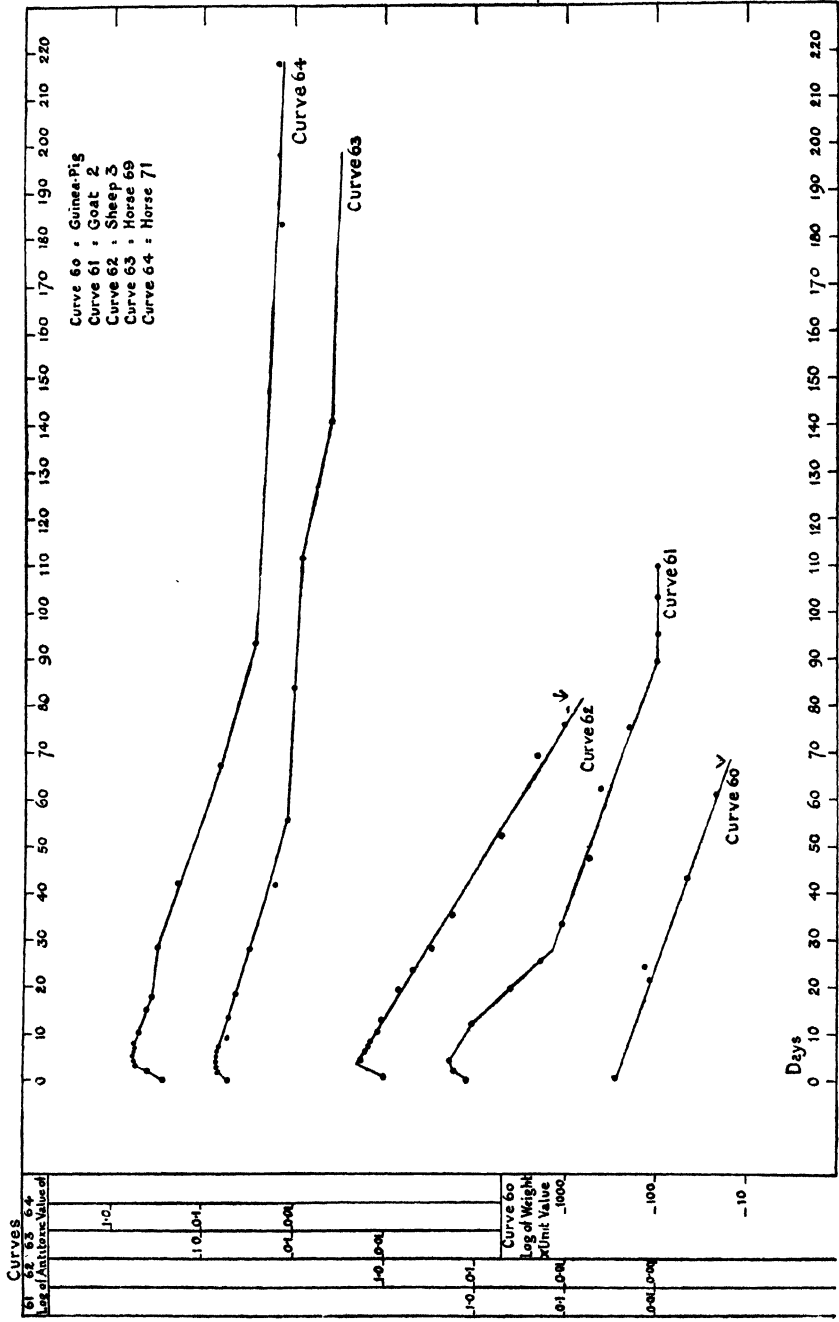


Chart XVI.

diluted because of the increasing blood volumes, Curve 60 on Chart XVI has therefore been constructed by plotting the log of "Weight \times unit value" (*i.e.* some fixed multiple of the total antitoxic content) against time. From this curve the rate of weekly loss of homologous antitoxin in guinea-pigs has been calculated as 25 per cent., *i.e.* of the same order as the rate of loss of homologous antitoxin in horses.

TABLE XXXII.

Showing the antitoxic value of a goat, a sheep and two horses at different intervals of time after the subcutaneous injection of unconcentrated horse serum containing diphtheria antitoxin.

Animal	Goat 2	Sheep 3	Horse 69	Horse 71	
Injection volume	10 c.c.	10 c.c.	50 c.c.	50 c.c.	
Total units	5500	7000	26,500	26,000	
Antitoxic value in units per c.c. at different intervals					
Interval	Unit value	Interval	Unit value	Interval	Unit value
Before injection	0.00	Before injection	0	Before injection	0.015
4½ hrs	0.20	2 hrs.	0.07	12 hrs.	0.45
6½ "	0.33	4 "	0.15	24 "	0.55
24 "	1.3	6 "	0.22	36 "	0.62
2 days	1.8	1 day	1.0	2 days	0.67
4 "	1.95	2 days	1.6	3 "	0.70
6 "	1.75	3 "	2.0	4 "	0.70
12 "	1.05	4 "	1.8	5 "	0.70
19 "	0.40	6 "	1.6	6 "	0.70
26 "	0.18	7 "	1.5	7 "	0.62
33 "	0.12	8 "	1.45	9 "	0.57
47 "	0.07	10 "	1.25	13 "	0.52
62 "	0.04	13 "	1.05	18 "	0.45
75 "	0.02	19 "	0.70	20 "	0.42
89 "	0.01	23 "	0.50	28 "	0.32
103 "	0.01	28 "	0.30	42 "	0.18
110 "	0.01	35 "	0.17	56 "	0.13
117 "	0.00	52 "	0.05	84 "	0.10
--	--	69 "	0.02	112 "	0.09
--	--	75 "	0.01	141 "	0.04
--	--	82 "	<0.01	201 "	0.033
--	--	--	--	278 "	0.030
--	--	--	--	--	--

Table XXXII records the antitoxic values of a goat, a sheep and two horses after the subcutaneous injection of horse serum containing diphtheria antitoxin. Samples of blood were withdrawn at somewhat irregular intervals. Table XXXIII has been compiled therefore to show the weekly rate of loss during successive intervals between the withdrawal of samples of blood. Curves for the antitoxic content of these animals and of the guinea-pigs recorded in Table XXXI are shown on Chart XVI (Curves 60 to 64). The two horses recorded both possessed a small amount of normal antitoxin and the curves recorded are therefore complicated by actively acquired immunity. Curve 63 consists of a continuous straight line from the first to the eighth week, with an average rate of loss of 20.2 per cent. per week; some slight natural stimulus intervened and for the next eight weeks the indicated loss, due to the differences between passive immunity lost, and active immunity gained, is extremely

rabbit had fallen below 10 per cent. in two weeks, but 10 per cent. of horse serum remained in both a goat and a sheep for three weeks.

TABLE XXXIV.

Showing the number of weeks taken for the antitoxic content of the animals recorded in Tables XXIX, XXXI and XXXII to fall below various stated fractions of the total amount injected.

Animal	Serum injected	1/10	1/100	1/1000
Rabbit 77	Rabbit	1½-2	5	8
Horse 980	Horse	11	24	38
" 981	"	9	20	30
" 69	"	8-12	—	—
" 71	"	9	—	—
Guinea-pig C, D, F	Guinea-pig	8	—	—
Goat 2	Horse	3	10	—
Sheep 3	Horse	3	9	—

SUMMARY.

1. The course of disappearance of passive immunity in rabbits injected with diphtheria antitoxin obtained from goats, men, guinea-pigs and cows, consists of the same three phases that follow the injection of horse serum.

2. The rabbits examined were more responsive to goat, human and guinea-pig serum than to horse and cow serum.

3. The course of disappearance of passive immunity in rabbits, horses and guinea-pigs injected with homologous antitoxin, consists of Phases A and B only, and Phase B is far slower than when heterologous serum is injected into rabbits.

4. Sheep and goats eliminate antitoxin obtained from a horse at a very slow rate, and Phase C is hardly detectable.

5. Natural immunity of horses to diphtheria toxin is gradually acquired by a number of increasing responses to external stimuli.

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THE NUTRITION OF BACTERIA, WITH SPECIAL REFERENCE TO *BACILLUS INFLUENZAE* (PFEIFFER)¹.

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(With Plate I.)

THE experimental work on which the paper is based was undertaken with the object of investigating the factors which influence the growth of bacteria in various culture media. The *B. influenzae* was selected as the best organism because of the comparative difficulty of obtaining growth on ordinary media, and the feebleness of the growth when obtained. The importance of food accessory substances, or "Vitamins" for the proper growth and nourishment of animals is generally recognised, and the influence of the same on the nutrition of bacteria has been occupying the attention of various investigators during the last few years.

LITERATURE.

Pfeiffer (1893) said the group was haemoglobinophilic, and considered the presence of haemoglobin necessary for growth. Grassberger (1898) could not obtain growth on media containing haematin except when *B. influenzae* was grown in symbiosis with other bacteria. Cantani (1901) succeeded in growing on media enriched with spermatic fluid, and he did not consider the presence of haemoglobin to be essential. Ghon and Preyss (1902) considered haemoglobin as necessary, even though it was present in very small quantities. They also obtained growth in association with other bacteria. Neisser (1903) similarly succeeded in growing on nutrient agar in symbiosis with *Xerosis bacillus* for twenty generations. Luerssen (1904) was successful in obtaining growth on haematin media to which dead cultures were added, but failed to get a growth in the presence of other living bacteria, as the *B. influenzae* was always overgrown by the associated organisms. Rivers (1920) obtained growth in haemoglobin-free media in symbiosis. Olsen (1920) got growth on haematin

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or haemin media in symbiotic relationship with other bacteria. Putnam and Gay (1920), and Stillman and Bourn (1920) failed to grow on haemoglobin-free media. Davis (1907) thought that haemoglobin was necessary, and showed that a very small amount was required. In a subsequent communication he (1907) considered two factors necessary, (1) haemoglobin which acts in very high dilutions, and (2) a factor present in animal and vegetable tissues. Fildes (1921) confirmed the observations of Davis that haemoglobin is essential but that it may be present in very high dilutions. He considered the iron in the blood pigment to act as a catalyst in accelerating the transfer of oxygen from the medium to the bacillus. He further stated that unaltered haemoglobin actually inhibits the growth. Rivers (1919) suggested the presence of some inhibitory substance in the human serum, as he obtained abundant growth on 5 per cent. fresh rabbit or cat blood, but not on human blood. Thjötta and Avery (1921) have confirmed the observations of Davis. They consider two substances essential. These are both present in blood, but also in other substances, *e.g.* vegetable material. One of these substances is resistant to heat, while the other is not. They have succeeded in growing *B. influenzae* in the complete absence of blood.

EXPERIMENTAL.

THE AMOUNT OF BLOOD REQUIRED FOR GROWTH.

Different proportions of sterile defibrinated fresh rabbit blood were added to 4.50 c.c. broth tubes ($pH = 7.6$). These were tested for sterility and then inoculated from a 48 hours blood broth culture of *B. influenzae*. The amount of growth developed was determined by (1) the degree of turbidity produced, (2) the relative number of bacteria in films stained with weak carbol fuchsin, and (3) the amount of growth obtained on blood agar tubes on subculturing. Table I shows that the highest dilution of rabbit blood that supported the growth was found to be 1-100,000.

One can hardly conceive that blood in such high dilutions plays the part of a nutrient material. Further experiments showed that if a watery solution, instead of nutrient agar was used no growth was obtained unless the blood was present in a dilution of at least 0.5 per cent. It seems, therefore, that the blood must be considered as an aid rather than the essential factor for growth.

Table I. *Relation of growth to concentration of blood.*

No.	Broth	Percentage of blood	Growth
1.	4.5 c.c.	0.01	+++
2.	"	0.001	++
3.	"	0.0001	+
4.	"	0.00001	+
5.	"	0.000002	-

Inoculum: One drop of the supernatant blood free portion of a blood broth culture.

DISTRIBUTION OF THE FACTORS IN BLOOD.

In order to determine in what constituent of blood or blood derivatives the accessory factor or factors are in greatest concentration a number of products were tested.

- (1) Ascitic fluid (human).
- (2) Blood serum (rabbit). Obtained from blood by repeated centrifugation.
- (3) Blood extract (rabbit). The blood was diluted half and half with sterile saline, was raised to boiling point in a water bath. The fraction separated from the clot was employed.
- (4) Solution of laked red blood corpuscles (rabbit). The corpuscles were laked with sterile distilled water.
- (5) Crystalline haemoglobin (sheep).

It will be seen from Table II that the greatest concentration of the factors necessary for growth is associated with the cellular fraction of the blood.

Table II. *Concentration of Accessory Factors in Blood Derivatives.*

No.	Dilution in broth	Ascitic fluid	Serum	Blood extract	Laked R. B. C.	Cryst. Hb.	Control
1.	1-2	.	+	+	+	+	.
2.	1-5	.	+	+	+	+	.
3.	1-10	.	±	+	+	+	.
4.	1-100	.	-	+	+	+	.
5.	1-1,000	.	-	+	+	+	.
6.	1-10,000	.	.	+	+	.	.
7.	1-100,000	.	.	-	+	.	.
8.	1-200,000	.	.	-	-	.	.

Inoculation from a blood broth culture, as above.

EFFECT OF HEAT ON THESE FACTORS.

Two drops of fresh rabbit blood were added to tubes of broth ($pH = 7.6$), and these were heated up to different degrees of temperature (Table III).

Table III. *Effect of Heat on Blood and Relation to Growth.*

No.	Broth	Blood factor	Growth
1.	4.5 c.c.	Unheated tube	+
2.	"	Heated at 60° C. for 3 hrs.	++
3.	"	Heated at 100° C. for 7 mins.	+++
4.	"	Autoclaved for 30 mins.	-
5.	"	" " + 0.5 c.c. serum	+
6.	"	" " + autoclaved serum	+
7.	"	+ 0.5 c.c. serum	-

Loop inoculation from a blood broth culture.

From these tables it is apparent that crystalline haemoglobin, or fresh blood plus plain medium is not a good menstruum for the growth of *B. influenzae*, though some growth in such media will occur. When blood is heated its value is decidedly increased, but when heated beyond a certain point the value is altogether destroyed. If fresh unheated serum be now added to this overheated blood it is reactivated, though serum by itself in the same pro-

portion does not support growth. If the serum be autoclaved its power of reactivating overheated blood is also destroyed.

This suggests that we are dealing with at least two different factors. The one present in autoclaved blood, highly resistant to heat, probably associated with the pigment, and the other heat labile present in the serum fraction of the blood.

Davis (1907) found that substances similar in their function to haemoglobin, viz. haemocyanine and haemoerythrine did not sustain growth. He was unable to find any growth inducing action in substances which readily gave up oxygen, such as hydrogen peroxide, and various organic and inorganic salts of iron, etc. Olsen (1920) tested various constituents of blood but all were ineffective. Haematin and haemin were tested in our experiments and they both failed to support the growth, but when fresh bacterial emulsions killed at 60° C. were added growth could be obtained. The inference from this is that growth inducing factors are also present in fresh bacterial emulsions.

SYMBIOTIC PHENOMENON.

It was pointed out as early as 1898 by Grassberger that close to the margin of colonies of staphylococci, the *B. influenzae* tended to form giant colonies. He also stated that if cultures of staphylococci were killed and mixed with the blood media the growth of *B. influenzae* was enhanced. We have often observed the more profuse growth of *B. influenzae* when it is cultivated on blood smeared agar in association with other organisms.

Experiments were carried out to study the phenomena with various types of organisms. A blood agar plate was inoculated with *B. influenzae*, and then at various points on the plate inoculations with other organisms were made. It is not necessary to detail these experiments as whatever type of organism was employed, similar results were noted in varying intensity. The plates showed that the colonies of *B. influenzae* close to the foreign colony are much bigger in size than those a little further away. There is seen a definite narrow area of inhibition, about $\frac{1}{2}$ to 1 mm. in size around the foreign colony. In this area none, or at the most a few colonies of *B. influenzae* develop. But beyond it is an area of augmentation of growth varying from 2 to 5 mm. in diameter, or at times more, in which the colonies of *B. influenzae* are very large and often crowded together, so as to give the appearance of "Satellitism" (see Plate I, fig. 1). Davis lays a great stress on this point; he claims to observe it in every experiment, and bases his diagnosis of suspected material on this observation. It was not found constant in our experiments, though at times it was extremely well marked.

The process is not dependent upon or due to haemolysis, and liberation of haemoglobin from the corpuscles, because it is observed equally well in blood laked or unlaked, and with haemolytic and non-haemolytic bacteria. No stimulating influence is noticed by seeding dead bacteria, but when the

dead cultures are intimately mixed with the medium the *B. influenzae* grows profusely.

It is clear that the associated bacteria during their growth alter the medium in such a way as to render it favourable for the growth of *B. influenzae*. This action may be due to:

- (1) Stimulating effects of certain products of bacterial metabolic activities.
- (2) Breaking up of the red blood corpuscles, and setting free the contents of the cells.
- (3) Destruction of an inhibitory substance normally present in blood.

To investigate these questions further experiments were carried out as under:

I. A few tubes were inoculated with *Staphylococcus albus*, 48 hours growth from agar was washed in sterile broth, sterilized in the water bath at 60° C., and 0.5 c.c. of this sterile emulsion was added to tubes of broth 4.5 c.c. ($pH = 7.6$). These tubes were tested for sterility, and then inoculated with *B. influenzae* from a young blood broth culture; controls with tubes of ordinary broth were kept.

The control tubes showed no growth but in the other tubes a growth of *B. influenzae* was obtained. The small amount of blood introduced from the culture tube could have had no influence in aiding growth, and this is further established by the absence of growth in the control tubes which contained an equivalent amount of blood. Similarly other organisms were tested and the same observations made (Table IV).

Table IV. *Growth of B. influenzae in plain broth + Sterile bacterial Emulsion.*

No	Broth	Emulsion	Organism	Growth
1.	4.5 c.c	0.5 c.c.	<i>Staphylococcus albus</i>	+
2.	"	"	<i>B. friedlander</i>	++
3.	"	"	<i>B. diphtheriae</i>	+
4.	"	"	<i>B. coli</i>	+
5.	"	"	<i>B. xerosis</i>	+
6.	"	"	Control	-

Inoculation from a blood broth culture.

II. In similar experiments using only Friedländer's bacillus and *B. diphtheriae* it was found that the organism could be subcultured to the twelfth generation, and live in each tube, as shown by growth on subculture, from 4 to 12 days.

III. The minimum amount of bacterial emulsion necessary for supporting the growth was next determined. The least quantity needed with the different organisms was:

1. Friedländer's bacillus	0.02 c.c.
2. <i>B. diphtheriae</i>	0.3 "
3. <i>B. xerosis</i>	0.2 "
4. <i>B. coli</i>	0.05 "
5. <i>Staphylococcus albus</i>	0.1 "

IV. *The growth inducing substances were found to be chiefly associated with the cellular fraction of the bacterial emulsion.*

A sample of bacterial emulsion (Friedländer's bacillus) was boiled for five minutes, the product centrifuged, and the clear supernatant fluid tested for the presence of growth factors. Their presence in great abundance was demonstrated, whereas in the supernatant fluid from a similar emulsion not exposed to heat, but allowed to remain at room or incubation temperature for days—only little growth inducing substances were present. This experiment shows that the growth inducing substances are set free from the bacterial cells into the surrounding fluid, the passage being quickened by heat. One exposure for five minutes does not completely remove these bodies, and small quantities can be extracted on the second and to a still smaller extent in the third treatment.

V. Autoclaving the emulsion, or heating it at 100° C. over a prolonged period destroys the substances.

VI. The heated emulsion can be filtered through a Berkefeld filter without loss of the growth inducing properties, and through glass-wool with only slight loss. If, however, the emulsion be first filtered through a filter paper, and then sterilized by filtering through a Berkefeld candle the loss is very considerable. Similar observations have been recorded by Lloyd (1916-17).

VII. Smearing the surface of a nasgar slant with the heated bacterial emulsion does not encourage the growth. But if it be incorporated in the medium active growth is obtained. The minimum amount of bacterial emulsion required in this case is much greater than was needed for liquid media.

From these experiments it seems to be established that substances which induce the growth of Pfeiffer's bacillus and which occur in the cellular fraction of the blood, are also produced during bacterial disintegration. They are resistant to a heat of 60° C., they stand boiling for five minutes, but are destroyed by boiling for fifteen minutes or by autoclaving. They pass through a Berkefeld filter unimpaired, but are lost to a great extent in filtering through paper. To induce growth small quantities only are required in liquid media, but comparatively large amounts in solid media. Simple smearing on to the surface of a solid medium is not sufficient, and incorporation into it is essential. They are associated with the body of the bacteria, just as they were in blood with the cellular fraction, and from the cells they pass out either by disintegration of the cells or by osmosis, and reach the *B. influenzae* by diffusion. In the case of liquid media, through the constant change in surface tension and electrolytic alterations, these bodies are easily brought into contact with the growing bacillus, but there being no such factors in the case of solid media incorporation into the medium itself is essential.

These experiments show that in the phenomenon of symbiosis the supporting bacterium helps growth by producing favourable substances during its active metabolism, and that these are diffusible substances that pass out and reach the main organism (in these experiments *B. influenzae*). This seems

to offer an explanation of the picture of satellitism sometimes observed. The growth factors passing out from the central colony of the foreign organism in all directions like the rays of light from a spot; the seeded *B. influenzae* lying in the way of these products, begin to multiply at the point of contact and to form colonies around the foreign colony. These products would naturally diminish in their amount and concentration the greater the distance from the foreign colony, and consequently the augmenting effect is observed only in a limited field round the foreign colony. Probably the products which are diffused from the foreign colony also produce certain alterations in the blood on the plate.

ALTERED BLOOD AND GROWTH.

We have seen that in the growth of *B. influenzae*, haemoglobin plays the part of an accessory food factor. It has been shown that though the growth in haemoglobin media attains a certain development, it is much greater when another bacterium is at the same time allowed to grow on the plate, or if the products of metabolism of the other organisms sterilized by heating at 60° C. be added to the media. It is apparent from these experiments that at least two factors are concerned which enhance the growth of *B. influenzae*. It has been mentioned previously that these two factors behave differently towards heat, one being heat stable and the other heat labile. The foreign colony not only furnishes the heat labile substance necessary but also breaks up the haemoglobin into its derivatives haematin, and haemin, and thus act in a double capacity with material advantage to the growth of *B. influenzae*.

That changes in the haemoglobin are important are evidenced by many experiments. Avery (1918) used oleic acid and sodium oleate in 1-1000 dilution to bring about changes in the blood, and got a good growth. In our hands this was not so successful. Mathews (1918) and McIntosh (1918) both make a tryptic digest of blood and claim to obtain a copious growth. In our experience the claim has not been established. Fleming (1919) secures abundant growth by using an acid extract of blood, but this also failed in our hands. Fildes' (1920) peptic digest gives a good growth, but the colonies are pin-point in size, and in this respect it is much inferior to the so-called "chocolate medium."

This chocolate or boiled blood medium has been extensively used by various investigators. We have already shown that heat, just like a symbiotic bacterium in some way either by liberation of some substance or by destroying any inhibitory factor in the blood renders it more efficient for the growth of *B. influenzae*. In order further to illustrate this point an experiment similar to the one described at the beginning of the paper was repeated with a few additions (Table V).

This table shows that the ox blood broth tubes heated to 60° C. for four hours, support growth of *B. influenzae* in greater dilution than those which were not so treated. Boiling the blood broth tubes for five minutes gave similar results. Besides the amount of growth in the tubes that were either heated or

Table V. *Concentration of blood, its relation to growth, and effect of heat.*

No.	Broth	Dilution of blood, Ox	Unheated	Heated at 60° C. for 4 hrs.	Boiled for 5 mins.
1.	4.5 c.c.	1-100	+++	+++	+++
2.	"	1-1,000	++	+++	+++
3.	"	1-10,000	+	++	++
4.	"	1-100,000	+	+	+
5.	"	1-200,000	-	?	+
6.	"	1-500,000	-	-	-

Inoculation from a blood broth culture. Results after 48 hrs. incubation.

boiled was much greater than in the unheated. With rabbit and guinea-pig bloods these results were not obtained, in fact heated tubes gave negative results in the same dilutions (higher only) in which the unheated blood gave definite growth. We cannot offer an explanation of the difference between ox and rabbit and guinea-pig bloods. There seems, however, no doubt that the growth inducing substances in rabbit and guinea-pig bloods are easily destroyed by heat. This suggests either that some inhibitory substance present in ox blood is destroyed by the heat, or that some growth inducing substance is liberated from the red cells. An explanation may be found in the inhibitory action on the growth of some bacteria by blood serum.

When graduated doses of serum were added to tubes of "chocolate" agar, it was found that the addition of large doses had a deleterious effect on the amount of growth of *B. influenzae*. It was further observed that this influence was not exerted to the same extent when the serum was previously heated for an hour at 55° C. Davis (1921) and Rivers (1919) have both observed this inhibitory phenomenon. The latter has shown that human serum is bactericidal to *B. influenzae*.

RELATION OF GROWTH TO HEAT AND TIME OF EXPOSURE.

It was found, when ordinary unheated blood was added to plain medium, the growth though definite was not abundant. When the blood was heated at 60° C. for an hour, growth was still scanty. But when heated at that temperature for a prolonged period, 4-6 hours, or at 80° C. for a shorter period, or at 100° C. for a few minutes, the growth was abundant. If submitted to a temperature below 60° C. even an exposure of many days does not render the blood suitable for growth, but generally makes it more ineffective. Exposure for half an hour to a temperature of 100° C. is still more deleterious, and autoclaving at 120° C. totally destroys the growth factors. We found that the maximum amount of growth was obtained with blood subjected to a temperature of 90°-100° C. for 10-15 minutes.

METHOD OF PREPARING A SUITABLE MEDIUM FOR *B. INFLUENZAE*.

In the preparation of this medium advantage is taken of the knowledge gained from the foregoing experiments. When blood is added to a tube of media and heat applied, the blood proteins coagulate, and the medium presents

a muddy, opaque appearance in the tube or poured plate. Filtration through ordinary filter paper by removal of the coagulated proteins produces a clear medium but one which is deficient in growth factors. Filtration through glass-wool produced a uniformly opalescent medium and this gives a luxuriant growth. The degree of opalescence can be greatly reduced by increasing the thickness of the glass-wool layer, without apparently producing any harmful effect on the medium.

After a series of experiments the following method was adopted, and in our hands it has proved of great value. Two important points came out in these experiments: (1) the reaction of the media, and (2) the degree of heat applied. In regard to the former definite growth was obtained between $pH = 6.2$ and $pH = 8.0$, but the optimum was $pH\ 7.2-7.5$. The heat applied must be just sufficient to extract all the growth factors from the blood. The nutrient agar after sterilization and addition of blood if previously adjusted to $pH\ 7.2$ gave the reaction $pH\ 7.4-7.5$.

The nutrient agar is prepared in the usual way, adjusted to $pH = 7.2$ and sterilized in large quantities. Blood, the source does not matter, sterile if possible, is obtained. The agar is melted in 200-500 c.c. quantities, and cooled to $80^{\circ}C$. While at this temperature the blood is added 1 part to 10 or 15 of media. This is well mixed and the temperature of the mixture gradually raised to $90^{\circ}C$., and kept at this temperature for 10 minutes. The mixture is filtered through a glass-wool filter which has been previously sterilized, the whole being kept hot. The filtrate is tubed immediately into sterile tubes, and one sterilization at $80^{\circ}C$. for 20 minutes is given.

One sterilization was as a rule found to be sufficient. If the temperature is raised to a higher degree the resultant blood agar is not so good. Both slant and stock tubes can be prepared. In the preparation of Petri plates care must be taken that the agar is poured into the dishes as soon as it has completely melted. If left in the bath even for a short time precipitation of the proteins takes place.

On this medium Pfeiffer's bacillus gives a slight growth in about 12 hours, and a very luxuriant growth in 48 hours. Colonies 4 mm. in diameter or even larger are sometimes seen. (See Plate I, figs. 2-4.)

In spite of this copious growth it was found that subcultures made after a week or so usually failed to give evidence of growth. This suggests either that the organisms are dead, or that during the copious growth they produce substances which are toxic to, or which at least inhibit growth even on a suitable medium. When these dead cultures and their products from the slant tube were smeared on the surface of a new slant of the same medium and this tube inoculated from a young culture, it was found that the number of colonies that developed were few whereas in a control tube the growth was abundant.

Thus it appears that on a suitable medium, the bacillus grows profusely, produces toxic matter in large quantities and itself dies. May it not be that a similar action takes place in the human body? If the soil is suitable, rapid

multiplication takes place, the toxic material possibly resulting from autolysis, is distributed throughout the body producing the severe symptoms, and the bacterium itself disappears. This view would explain the contradictory statements as to the presence or absence of *B. influenzae* in epidemics.

EFFECT OF FRESH TISSUE AS A SOURCE OF GROWTH FACTORS.

From the experiments already recorded, it is clear that the augmentative effect on the growth of *B. influenzae* in its symbiotic relationship with other bacteria, is associated with the active metabolic processes of living organisms. A drop of fresh blood cannot take the place of these living organisms, but a piece of sterile animal tissue well washed in saline was found to have this stimulating property. If the fresh tissue be autoclaved it loses its power of augmenting growth. Thus this growth factor in the fresh tissues is heat labile, and is probably allied in nature to the substance produced during bacterial symbiosis. This substance is of a diffusible nature, it is not blood, it occurs only in the fresh tissues, is heat labile, and for the growth of *B. influenzae* is needed only in small quantities.

From the clinical point of view the general statement has been made that in mixed infections *B. influenzae* is more dangerous and pathogenic to animals, and possibly to man. We have shown that living tissues will stimulate the growth of Pfeiffer's bacillus quite as actively as associated bacteria. This augmenting effect is being exerted by the tissues of the body to an unlimited extent, and in our opinion the additional influence of an associated organism in the respiratory passages would not make any very great difference in the final results, unless it be that the associated organism favours growth by removing an inhibitory or antagonistic substance in the tissues or plasma.

The same stimulating effect was demonstrated with fresh vegetable tissues. If pieces of carrot or potato were put on blood agar plates, the colonies of *B. influenzae* are crowded round these bits of vegetable tissues, and are much larger in their vicinity than the colonies in other parts of the plate. Pieces of vegetable tissues that were autoclaved or otherwise heated at a high temperature for a few minutes failed to show this augmenting effect. Fresh vegetables contain the various vitamins, which are of such vital importance in animal growth and nourishment, and probably similar substances are concerned in bacterial nutrition as well. As yeast is rich in some of these vitamin bodies, we attempted to get these growth inducing substances from it.

THE HEAT LABILE FACTOR.

Some yeast extract was prepared from a sample of dried commercial yeast. To 80 gms. of yeast were added 300 c.c. of distilled water, and 10 c.c. of *N*/10 hydrochloric acid to make it slightly acid. This was boiled over a flame for 10 minutes and the flask allowed to stand overnight. The clear supernatant fluid was pipetted off the following morning, reaction adjusted to $pH = 7.2$, and sterilized in three different portions. One portion by autoclaving at $120^{\circ}C$.

for half an hour, another by three days' fractional sterilization, and the third by filtering through a Berkefeld candle.

Graduated amounts of each sample were added to tubes of ordinary broth, and inoculated with *B. influenzae*. The broth tubes containing yeast extract sterilized by heating or autoclaving gave negative results, while in the third portion sterilized by filtering through a Berkefeld candle growth was obtained in slight amounts up to a dilution of 1-250. The bacillus survived for three or four days, and in such broth tubes died out in the third subculture. Similar experiments with a sample of brewer's yeast showed it to be definitely richer in the yield of growth factors. In this case growth was obtained up to a dilution of 1-1000, and subcultures up to the fifth generation.

If the primary inoculation was made from a blood broth tube, the growth survived longer than if made from a blood agar tube where the needle touched the surface only lightly. It suggests that in the former case sufficient of another factor was carried from the blood broth tube. This factor present in the blood, the "X" factor of Thjötta and Avery (1921), we have seen previously needs to be present only in very high dilutions, and is heat resistant. The other factor "V" which is heat labile as seen from absence of growth in heat sterilized yeast extract, has already been demonstrated in red blood corpuscles, bacterial extracts, and in animal and vegetable tissues.

Hopkins (1912) has observed that vitamins are substances readily absorbed from solution. Lloyd (1916-17) has also pointed this out. Thjötta and Avery (1921) found that the growth inducing substances are absorbed from yeast extract by bone charcoal, and that the process of absorption is accelerated by the agency of heat. Our results are in agreement with these observations.

Further study of the "V" factor was continued with vegetable extracts, potato, tomato, and carrot. Similar experiments as with yeast extract were carried out, and it was observed that potato, and carrot extracts were decidedly superior to the yeast extract in their content of growth inducing substances (Table VI).

Table VI. *Comparison of Yeast and Vegetable extracts.*

No.	Dilution in broth	Yeast	Potato	Carrot	Tomato
1.	1-5	++	++	++	++
2.	1-10	++	++	++	++
3.	1-100	+	++	++	+
4.	1-1,000	+	++	+	+
5.	1-10,000	-	+	±	-

Inoculation from a yeast extract broth culture.

THE HEAT STABLE FACTOR.

We have seen that this is present in the blood associated with the cellular fraction and the pigment. By itself it is not capable of supporting growth, but the amount needed when in association with the other factor is very very small. Olsen (1920) states that the Tincture Guaiacum, and the Benzidine tests for blood, go hand in hand with the ability of blood derivatives to support

growth. Thjötta and Avery have confirmed these observations, and in our experiments we have found the same parallelism. The latter two investigators have observed a positive reaction with potato, and its capacity to support growth in the complete absence of blood. When studying the heat labile factor it was noted that potato extract was superior to the yeast extract, perhaps it is on account of the former possessing both the factors.

Its Presence in Vegetable Tissues.

Potato contains various peroxides, and the two vitamins A and B, amongst many other undetermined factors. One has often observed that scrapings of fresh potato exposed to air rapidly change colour, as a result of oxidation processes. Hydrogen peroxide is rapidly oxidised with liberation of gas bubbles, and if the benzedine reaction as used for blood is applied, a bluish-purple colour is given. If potato be heated or autoclaved the benzedine test is negative, and the potato no longer supports growth. This suggests that the enzymes concerned with the oxidising processes are more or less associated with the heat labile factor, and that the X factor of Thjötta and Avery is not connected with them. This leads one to attach less importance to the benzedine reaction, for although it is true that in the case of blood there is a parallelism between the benzedine reaction and the yield of growth, this is not the case with vegetable tissues. Moreover, we find that many other vegetable tissues which do not give this test support the growth.

An alcoholic extract of potato and carrot was prepared by triturating the vegetable with sterile sand, and alcohol was added in the proportion of an ounce to 5 gms. of the vegetable. It was left overnight, the supernatant alcoholic solution was pipetted off, the alcohol evaporated over a low heat, 45° C., and the resultant syrupy fluid taken up in sterile distilled water, and the whole sterilized by filtration through a Berkefeld filter. A watery extract was also prepared, by boiling the triturated vegetable for five minutes, the resultant fluid being sterilized by filtration through a Berkefeld filter.

Both the alcoholic and the watery extracts were found to support the growth of *B. influenzae* in the complete absence of blood. Half cubic centimetre quantities of the extracts were added to tubes of broth 4.5 c.c. There was always more growth in the tubes containing the alcoholic extract than with the watery extract, and still more in those with both the extracts. It is apparent that the growth inducing substances are extracted both by water and alcohol from its complex combination in these vegetables, alcohol being the better of the two. But it was found that all the growth requirements for a prolonged activity are not present in either of the two extracts, while the two of them in combination seem to contain most that is needed. When these extracts are added to tubes of melted agar growth is again supported. Experiments similar to those carried out with bacillary extracts were repeated. For want of space the various tables are not reproduced, and just a summary of these experiments is appended.

1. Alcoholic extract of potato	Growth in a tube from 3-7 days	Cultivated up to 6 generations
2. Watery extract of potato	Growth in a tube from 3-5 days	Cultivated from 4-6 generations
3. Alcoholic extract + Watery extract of potato	Growth in a tube from 3-11 days	Cultivated from 8-10 generations
4. Alcoholic extract of carrot	Growth in a tube from 3-6 days	Cultivated from 4-5 generations
5. Watery extract of carrot	Growth in a tube from 3-4 days	Cultivated from 3-4 generations
6. Alcoholic extract + Watery extract of carrot	Growth in a tube from 3-8 days	Cultivated from 6-7 generations

Although in such tubes the bacillus survived up to a number of days and could be cultivated by loop inoculation from a tube to tube to a number of generations, ultimately it died out, manifesting the want of some other product which had probably not been extracted with either of the two extractives. Thjötta and Avery use these vegetables in the raw state putting sterile pieces into tubes of broth. Their technic was followed and the experiments repeated. It was noted that in this case the bacillus could be carried from tube to tube by loop inoculation every 48-72 hours indefinitely up to, at any rate, the twenty-first generation when the experiment was discontinued. On the average in any one tube the bacillus was living from 8-18 days. The potato evidently supplies all the needs for the nourishment of *B. influenzae*.

Broth is a preparation from animal tissues, and may have various substances present in it. In order to eliminate their possible influence, tubes of Ringer's physiological solution were prepared, and to these sterile pieces of potato were added. The bacillus grows and multiplies in such a medium, and subcultures can be carried on indefinitely. But in any single tube it lives for a shorter period 8-11 days. Sterile pieces of potato added to tubes of normal saline also support growth, the bacillus living only from 5-6 days in such tubes.

The above experiments throw light on a number of interesting points, for example, it shows that potato contains all the substances needed for growth. Not only does it supply the heat labile, and the heat stable factors, but it also provides the proteins, carbohydrates, and other products of protein hydrolysis, such as aminoacids, and free nitrogen. It is interesting to note that *B. influenzae* is capable of obtaining all its nitrogen requirements from the native proteins of potato. Then, again, we notice that the bacillus lives for a longer period in tubes of broth plus potato, than in Ringer's solution plus potato, showing that the meat extracts in the broth prolong the duration of life; also the salts in Ringer's solution enable the bacillus to survive over a longer period than in saline plus potato.

Another very important point is in reference to the reaction of the medium. The optimum for growth was established at pH 7.2-7.5, the natural reaction of potato is acid to phenol red pH 6.2-6.5. The reaction of broth (pH 7.6) was

never readjusted after the addition of potato, and it left the final reaction slightly acid, pH 6.4-6.7. The bacillus grows abundantly at this pH value and no harmful effects of this acid reaction were noticed. It is likely in the presence of raw substances the reaction is not of great importance, perhaps on account of the presence of some "buffers" which mitigate the unfavourable effects.

ACTION OF HEAT ON POTATO.

When these tubes of potato broth were autoclaved, and tested for growth-supporting properties, no growth was obtained. But when the destroyed heat labile factor was replaced by the addition of yeast extract, which we know only contains the heat labile factor, a fair amount of growth was obtained, but it was not as luxuriant as in the original tubes. It shows that the heat stable factor was present in the autoclaved potato, and that the heat labile factor restored by the addition of yeast extract is not suitable, or else that there has been destroyed by autoclaving besides the heat labile factor, something in the potato which cannot be made good in the added yeast extract. Perhaps one of the many enzymes, but one cannot say what rôle these ferments play in the growth of the bacillus in plant tissue media.

Other Plant Tissues and Growth.

Other vegetable tissues were similarly tested for growth-supporting properties. Banana, apple, and white turnip were employed. All the three gave good growth and supported the growth in subsequent generations. There are slight differences in the amount of growth yielded, thus apple is not as good as banana, and this not so good as turnip, whilst they are all inferior to potato. But these results are not strictly comparable, as they do not refer to any weight relation of the various vegetables used, yet they are of interest to prove that *B. influenzae* can be grown in the complete absence of blood and blood derivatives. Besides they are of importance in emphasizing the relation of plant tissues to bacterial growth and nutrition.

ADDITION OF PLANT TISSUES TO BLOOD MEDIA.

On addition of watery or alcoholic extract of these vegetables to melted blood agar no stimulating effect was noticed. If, however, solid pieces of the raw tissues were added to tubes of blood broth there was a definite augmentative effect on the growth. The growth in such cases is first observed in the close proximity of the tissue pieces in the depths of the tube, rather than at the surface where the supply of oxygen is most abundant. This is of interest and importance; the organism grows where there is an abundant supply of the various enzymes, and growth factors, probably there is also a transference of oxygen from the tissue cells.

DISCUSSION.

It has been demonstrated that in the growth of *B. influenzae* haemoglobin in the high dilutions necessary to induce growth, does not play the part of a nutritive substance, but acts in some way as an accessory factor. Thus, it may

act as a catalyst, quickening the reaction velocity of proteolytic metabolism, and rendering an easy and ready supply of nitrogenous substances. The fact, however, that it acts irrespective of the nature of the medium seems to negative this suggestion. We found, for example, that Tryptamine agar supposed to be rich in free aminoacids was not superior to ordinary agar. It may, however, be supposed that haemoglobin acts as a catalyst, and that this action may be dependent in some way upon the iron in the haemoglobin molecule, but the iron itself cannot be said to be more essential than any other element, as various organic and inorganic compounds of iron tested by Davis were valueless. One may consider that the function of haemoglobin in the body, as a carrier of oxygen, gives to it the power of supporting growth. But this is evidently not so, since heating destroys its usefulness as a carrier of oxygen, but increases its capacity to stimulate growth. Again haemocyanin performs the similar function of carrying oxygen but does not support growth.

It has been shown that there are at least two distinct factors in blood which by their combined action stimulate the growth of *B. influenzae*, either of them alone being insufficient. Probably one of them is haematin, which contains the pigment and is associated with iron. The second factor is a heat labile substance, which is readily absorbed from solution, and passes a Berkefeld filter unimpaired, but its nature we do not know. Fildes raises the question as to the possibility of the second factor being a peroxide of such a nature, that through the catalytic action of haematin the transference of oxygen to the bacillus from the peroxide and the medium is accelerated. We observed in tubes of blood broth to which pieces of raw potato were added, that the growth was extensive in the vicinity of these tissues. The fresh potato contains peroxides, and various other enzymes, but it does not necessarily follow that it is the transference of oxygen by the catalytic action of haematin which aids the growth. There is sufficient evidence against this assumption in the fact that heated blood gives luxuriant growth, and that the temperature to which it is subjected alters if it does not actually destroy the peroxides. Further, autoclaved potato, which does not give the peroxide reaction, plus yeast extract, in which the peroxides are absent, supports growth, again in synthetic media in which there is no haematin to exercise a catalytic action growth is also obtained.

In the raw state the vegetable tissues contain the peroxides and various enzymes and ferments. They also contain the fat soluble A, the water soluble B, and the C vitamins, in greater or less proportions. We know these three vitamins, but probably there are many more whose value we have not so far appreciated, and which may play an important part in bacterial nutrition. One thing is certain that the growth inducing substances, like the vitamins, are associated with living and active tissues, both plant and animal. A heat labile factor corresponding to the one in blood, has been demonstrated in bacterial metabolic products, yeast, and in fresh animal and vegetable tissues. The second factor which is heat stable has been found to exist in various plant tissues as well as in blood.

Thus it seems that these growth inducing bodies comprise a large group of substances, occurring in blood, in bacterial products, in animal and in vegetable tissues. Probably the nature varies with the source, but they all seem to have certain properties in common, and they all act as substances accessory to the other food constituents. Their mode or mechanism of action is not understood, and their chemical nature not known, the criteria for their identification being so inadequate. It is suggested that the growth stimulating properties are related to the presence of certain oxidising, and reducing enzymes in fresh plant tissues, as well as to the presence of vitamins.

CONCLUSIONS.

1. Rabbit blood in 1-100,000 dilution supports growth.
2. Haemoglobin in such high dilutions acts as a catalyst in some essential process, rather than as a nutritive agent.
3. The growth depends upon two distinct and separate factors, a heat labile, and a heat stable, which occur in highest concentration in the cellular fraction of the blood.
4. Other organisms distinctly favour the growth of *B. influenzae*.
5. Growth can be obtained up to a number of generations in plain broth plus sterile bacterial emulsion, in the absence of blood.
6. The emulsions can be heated at 60° C. for many hours, or boiled for a few minutes, and filtered through a Berkefeld filter, without impairment.
7. The bacillus grows profusely on a heated blood medium.
8. The augmentative effect is also exercised by fresh animal and vegetable tissues.
9. Sterile raw potato and many other plant tissues, apple, banana, turnip, and carrot, contain both the factors and can replace blood and animal tissue extracts in culture media.
10. The bacillus is not essentially an haemophilic organism but requires certain bodies to support its growth. These are of the nature of hormones, but the way in which the different factors operate to promote the growth is not understood.

In concluding I wish to record my sincere thanks to Professor Beattie for his kind help, valued criticism, and his permission for publication.

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DESCRIPTION OF PLATE I.

- Fig. 1. Symbiotic phenomenon. 54 hours' growth on blood smeared agar plate, with a foreign colony of *Staphylococcus albus*. × 6.
- Fig. 2. 54 hours' growth of *B. influenzae* on ordinary blood smeared agar plate. × 6.
- Fig. 3. 24 hours' growth of *B. influenzae* on blood agar, prepared as described in the text. × 6.
- Fig. 4. 5 days' growth on the same medium as in Fig. 3. × 6.



Fig. 1

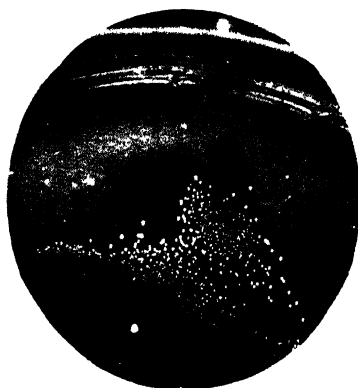


Fig. 2

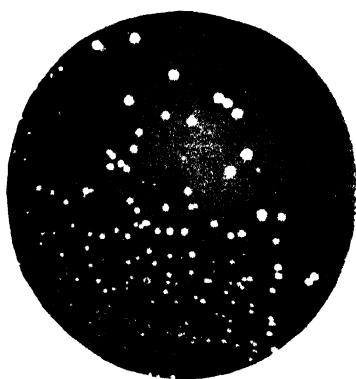


Fig 3



Fig. 4

AN OUTBREAK OF PORK PIE POISONING AT DERBY.

By C. F. PECKHAM.

(Assistant Bacteriologist to the Derbyshire County Council.)

WITH A FOREWORD BY
WILLIAM G. SAVAGE, M.D.

FOREWORD.

THERE are so many unexplained problems in connection with food poisoning that it is still of importance to study in detail and with care every outbreak. I have perused the records of several hundred food poisoning outbreaks, published and unpublished, and the prevailing impression left is one of regret that so many opportunities of clearing up obscurities and elucidating channels of infection have been lost by omission to push investigations home. The outbreak recorded here is in pleasant contrast and worthy of detailed study. It is noteworthy for two features. The bacillus isolated was not only isolated from the incriminated food but it was recovered from water from a tank in the slaughter-house, a rather unusual feature. This suggests a method of infection which has not hitherto been reported.

The other feature of interest is the serological character of the bacillus isolated. By the courtesy of Mr Peckham both cultures have been sent to me and Mr Bruce White has extensively studied its qualities in connection with extended investigation which we are making on the serological relationships of the Salmonella Group. We are not only in a position to confirm the very careful work of Mr Peckham but the strain (since both the pie and tank bacilli are identical) is unique in its serological relationship. It is identical with a strain isolated by us in May 1922 from a pig suffering from swine fever but otherwise, so far as we are aware, they stand alone. The two strains belong to no recognised type since serologically they stand between *B. enteritidis* and the larger *B. aertrycke*—*B. suispestifer* group. Hitherto no such intermediates have been described so the outbreak is of special interest.

W. G. S.

The following account of an outbreak of food poisoning, due to the eating of pork pies, is interesting because a bacillus of the Gaertner-paratyphoid group was isolated from the remains of a pork pie and from a tank of water on the same premises where the pork pies were made.

The two strains of bacilli were identical in all their reactions and closely resembled *B. Gaertner*. They could only be distinguished from *B. Gaertner* by absorption tests as shown later in the Report.

The pies were made in Derby and cases of poisoning occurred in Derby, Belper and Ambergate: the total number notified being 37.

The chief clinical signs were pains in the abdomen and limbs, violent vomiting and diarrhoea and pyrexia. There were no deaths, but in some cases the illness was severe and lasted for three weeks.

The period of time between eating the pies and onset of illness varied from 1½ hours up to 21 hours, the average time being approximately 11 hours.

Sausages, polony and potted meat were also purchased from the same source by two families in which illness occurred. In one case mother and daughter had pork pie and were ill: the father had only polony and suffered no ill effects.

The second family had pork pie and one hour before onset of illness had sausages. Two children of this family had potted meat only and sickness began 37½ hours after. The potted meat was eaten at 8.30 p.m. and again at 9 a.m. next morning. No other cases were notified after eating potted meat and it is probable that in this case the potted meat had become infected in the house, *i.e.* using the same knife for serving both pie and potted meat.

INSPECTION OF THE PREMISES.

By Dr WRAY, *Assistant Medical Officer of Health*, and
Mr HANSON, *Meat Inspector (Borough of Derby)*.

These premises were visited on October 11th, 1921, and comprise a pork pie and bacon factory: the number of employees being five males and one female.

The pigs are slaughtered in the slaughter-house. Offal is removed and gut scraping is carried out in the slaughter-house. The offal is then placed in a tank.

Carcases are taken by overhead trolley to a cutting up room. At one side of this room is the cutting block, and opposite to this are boilers or cookers and a mincing machine.

Make up of pies.

Make up room. At the end of this room, which is between cutting up room and cooking room, remote to the door leading to the cutting up room and yard and slaughter-house, is a bench where the pastry is brought and the pork filled in by the female employee, who alone handles the pork after it is cut up for pie making.

On Friday, October 7th, two hundredweight of pies were made, and these were distributed and sold on October 8th.

Cooking room. The pastry is made by the female worker on a bench in the pie cooking room. It is then conveyed in pans to the make up bench in the make up room described. The jelly for filling is made by the same firm and only sufficient is made to carry on from day to day.

The temperature of the ovens is stated to be 400° Fahr. (but the proprietor admits his thermometer is not accurate). No one handles the pies

except the female worker. When cooked they are placed on a bench in the middle of the cutting up room to cool: when cooled sufficiently the jelly is poured into the pies through a vent hole in the top crust of the pie. They are then distributed to the various shops on trays by a motor van. The proprietor says there is no possibility of under-cooking. All his ovens had been cleaned out and fires attended to the week previous.

Utensils. The baths, basins and carry tins are scrubbed out each day. One domestic bath in slaughter-house and one in cutting up room are provided with plugs, but are not provided with channel outlets, consequently they are emptied by lading with a small tin: hence water infected may thus remain.

The various utensils on both days of inspection appeared clean except the bath in the slaughter-house in which the offal is placed.

Duties of employees. The female worker as stated above. One man is supposed to handle the cut carcass. One is supposed to handle the offal. The others carry the various portions of the carcass to the curing room downstairs, and also attend the boiling, etc. It is doubtful if in practice these men are kept strictly to these duties. It is more probable that the male employees do whatever work is required at the moment.

Health of employees. They all claimed perfect health and stated that there was no illness in any of their families or in the houses in which they reside.

Condition of buildings.

Slaughter-house: Clean.

Pens: Fairly clean, but malodorous.

Cutting up room: Benches clean; floor, which was a wooden one, sticky and covered with wood chips.

Cook house: Clean.

The factory was again visited on October 19th, 1921. Slaughtering had taken place. The offal was in the bath mentioned. This bath on both occasions was not clean: the sides were dirty and sticky with dark deposits.

The following specimens were taken and sent to this laboratory:

1. Pie meat (uncooked).
2. Cold jelly (before boiling for pies).
3. Jelly (after boiling).
4. Lard.
5. Water from tank (or bath) in which large intestines were placed after slaughter.

The jars in which the specimens were placed had been previously boiled and wrapped in sterilised lint.

My thanks are due to Dr A. E. Brindley, Medical Officer of Health, Borough of Derby, for allowing me to use the above extracts from his report.

BACTERIOLOGICAL INVESTIGATION OF THE CAUSE
OF THE OUTBREAK.

On October 10th, I received from Dr Allen of Belper a portion of pork pie, purchased from a shop in Derby on October 8th, being one of a batch baked on October 6th, and alleged to be the cause of an outbreak of food poisoning involving at least 37 people. The pie was apparently fresh and had no offensive odour. The jelly was firm but was found to contain small whitish specks which turned out to be colonies of bacteria, when examined with the aid of a lens.

Emulsions made from the jelly and meat were plated out on bile salt lactose agar and lactose litmus agar. It was impossible to get a piece of meat that had not been in contact with the jelly. After 24 hours' incubation at 37° C. bacteria giving the following morphological and cultural reactions were obtained from both meat and jelly:

A small actively motile Gram negative bacillus, turning litmus milk acid, then alkaline, forming acid and gas in serum water containing glucose, dulcitol, salacin, mannite, maltose, galactose, and laevulose. No change in saccharose, lactose, glycerine, inulin and raffinose. No indol was produced in peptone water and neutral red was reduced.

Typical *B. coli* were present in fair numbers.

On October 19th I received from the same factory the following specimens: lard, cold jelly, boiled jelly, uncooked minced meat, a pork pie, and water from a tank in the slaughter-house. These were examined by lactose bile salt agar plate culture with the following results:

Lard. A few colonies of *B. coli*.

Cold jelly. *B. coli* and moulds.

Boiled jelly. *B. coli* and *Staphylococci*.

Uncooked minced meat. *B. coli* and *Enterococci* were found in large numbers.

There were a great many other bacteria present, but no organisms of the paratyphoid group were found.

Pork pie. Moulds. *Staphylococci* and a coliform bacillus were isolated. No organisms of the paratyphoid group were found.

Water from the tank. This water was distinctly coloured with blood and contained a small quantity of suspended matter. Plates of lactose bile salt agar were sown before and after incubation, and a bacillus, identical in morphological and cultural characteristics with that obtained from the portion of pie sent from Belper, was obtained in large numbers. A small portion of the incubated tank water was inoculated into a guinea-pig which died in 18 hours, and a bacillus, identical to that already isolated from tank water and pork pie, was obtained from the heart and spleen in pure culture.

No specimens of faeces from patients who had partaken of pork pie from this batch were received, but blood from three of them who suffered from

the poisoning was tested on the third day of illness, and in each case agglutinated the pork pie bacillus, the tank bacillus and that recovered from the inoculated guinea-pig in dilutions carried up to 1-100.

Blood from a fourth patient "A," obtained 17 days after eating a pork pie of this batch was tested with the above cultures and agglutination reached 1-500.

From the cultural characteristics of these bacilli, it was evident that they belonged to the Gaertner-Paratyphoid group and serological tests were undertaken to determine their place in this group. For this purpose the following cultures and specific sera were obtained from the National Collection of Type Cultures, Lister Institute: *B. paratyphosus* B. Strain "Tidy"; *B. suispestifer*. Strain "Mutton"; and *B. enteritidis* Gaertner, Strain "Stokes."

Direct agglutination tests with the blood of patient "A" (the only available blood at the time) were made, with the following result:

Table I. *Agglutination tests with serum of patient "A."*

Microscopical—2 hours.

Culture	1-100	1-250	1-500
<i>B. paratyphosus</i> B.	+	+	+
<i>B. suispestifer</i> *	+	+	+
<i>B. Gaertner</i>	+	+	+

The result was of such an indefinite character that an absorption test was made, and the result is shown in Table II:

Table II. *Agglutination tests after absorption.*

Microscopical—2 hours.

Serum and absorbing strain, 2 hrs. at 37° C.	<i>B. paratyphosus</i> B.		<i>B. suispestifer</i>		<i>B. Gaertner</i>		Control
	1-100	1-500	1-100	1-500	1-100	1-500	
Patient "A" serum } <i>B. paratyphosus</i> B. }	-	-	+	+	-	-	-
Patient "A" serum } <i>B. suispestifer</i> }	+	-	+	+	-	-	+ *
Patient "A" serum } <i>B. Gaertner</i> }	+	-	+	+	-	-	-

* These two tests gave very unreliable results. It was found that the cultures in use had gone "rough." Fresh cultures were obtained, but could not be tested with the above serum owing to the serum being used up.

A test was made to endeavour to find out how much of the agglutinins could be absorbed from specific sera by this "tank" bacillus. This is shown in Table III as follows:

Table III.

Serum and culture used for agglu- tination tests. Microscopic 2 hours at 37° C	Sera absorbed with "tank" bacillus, 2 hours at 37° C						Unabsorbed serum controls			Controls
	1-200	1-500	1-1000	1-2000	1-4000	1-8000	1-2000	1-4000	1-8000	
<i>B. paratyphosus</i> B.	+	+	+	+	+	-	+	+	-	-
<i>B. suispestifer</i>	+	+	+	+	-	-	+	-	-	-
<i>B. Gaertner</i>	+	+	+	+	-	-	+	+	+	-

In the above test saline emulsions of 18 hours old cultures were used, and by this method suitable suspensions were obtained. It will be seen that the

bacillus under examination is not *B. paratyphosus* B. or *B. suispestifer*, but is closely allied to *B. Gaertner*. As will be seen from this table, the "tank" bacillus was able to absorb a considerable quantity of the *B. Gaertner* agglutinins. Having some of this absorbed serum left over, it was again saturated with "tank" culture, to see if it was possible to lower the titre still further. A second test gave the same result.

The tank bacillus was inoculated into a guinea-pig on several occasions, using killed bacilli, and from it serum was obtained and was used for direct agglutination and absorption tests by the macroscopic method.

Table IV shows the agglutinating power of this serum for the chief members of the food poisoning group:

Table IV.

Culture	Dilution of serum							Controls
	1-20	1-100	1-200	1-500	1-750	1-1000	1-1500	1-2000
"Tank" bacillus	+	+	+	+	+	+	+	±
<i>B. Gaertner</i>	+	+	+	+	+	+	+	+
<i>B. paratyphoid</i> B.	-	-	-	-	-	-	-	-
<i>B. suispestifer</i> No. 1	+	-	-	-	-	-	-	-
" " No. 2	+	-	-	-	-	-	-	-

Two fresh strains of *B. suispestifer* were obtained from the Lister Institute and were found to be satisfactory.

The "tank" bacillus and the bacillus obtained from the pie had been re-plated and single colonies again re-plated several times, and tested out with sera but no change had taken place in their agglutinating powers.

The "tank" bacillus serum was absorbed with *B. paratyphoid* B. and *B. suispestifer*, but no change in agglutinating power for the "tank" bacillus took place.

Table IV shows the "tank" bacillus to be *B. Gaertner* or a bacillus very closely allied. To settle this point an absorption test was performed and is shown in Table V:

Table V. Absorption tests with serum of guinea-pig inoculated with "tank" bacilli.

Absorbing culture and test culture	Dilution of serum					Controls
	1-100	1-400	1-800	1-1600	1-2000	
Serum absorbed with <i>B. Gaertner</i> and tested with "tank" bacillus	+	+	+	+	±	-
Serum absorbed with "tank" bacillus and tested with <i>B. Gaertner</i>	-	-	-	-	-	-

In Table III it will be noticed that it was impossible to absorb the agglutinins from a Gaertner serum by the aid of the "tank" bacillus. Tables VI and VII confirm this result:

Table VI. Agglutination tests with *B. Gaertner* serum.

Test organism	Dilution of serum					Controls
	1-100	1-500	1-1000	1-2000		
"Tank" bacillus	+	+	±	±	-	-
<i>B. Gaertner</i>	+	+	+	+	-	-

Table VII. *Absorption and agglutination tests with B. Gaertner serum.*

Absorbing culture and test culture	Dilution of serum				Controls
	1-100	1-500	1-1000	1-2000	
Absorbed with "tank" bacillus and tested with <i>B. Gaertner</i>	+	+	+	+	-
Absorbed with <i>B. Gaertner</i> and tested with "tank" bacillus	-	-	-	-	-

A number of the foregoing tests were also done with the bacillus isolated from the pork pie and the results were the same as those given by the "tank" bacillus.

In connection with this work a small investigation was made to see if the tanks used in other pig slaughter-houses contained any organisms of the Gaertner-paratyphoid group, and on November 11th samples of tank water from two pig slaughter-houses in Derby were submitted for examination. The samples were received in sterile test tubes and each was slightly blood stained. Nothing was added and they were incubated over-night. After incubation they were plated on bile-salt agar, and any suspicious colonies that developed were examined for motility and preliminary agglutination tests with anti-sera.

The result of this examination was that from one of the tanks a bacillus was isolated which gave all the biochemical and serological reaction of *B. Gaertner* as shown in Table VIII:

Table VIII. *Agglutination tests with bacillus from tank.*

Serum	1-200	1-1000	Controls
<i>B. Gaertner</i>	+	+	-
<i>B. supestifer</i>	-	-	-
<i>B. paratyphosus</i> B.	-	-	-

The tank water from the second slaughter-house was rich in excretal organisms, but although a large number of colonies were tested, none were found which reacted to any of the tests applied to the Gaertner-paratyphoid group.

CONCLUSIONS.

It can be fairly safely assumed that the causal organism of the outbreak was the bacillus isolated from the pork pie. Agglutination tests with the sera of the patients who had partaken of that, or pies of the same batch, show this by their positive reactions to this bacillus and the bacillus isolated from the tank water.

It is to be regretted that no specimens of faeces from these patients were sent for examination.

That the pies were infected from the tank water is fairly conclusively proved by the isolation of a bacillus identical in all respects with the bacillus obtained from the pie, and this bacillus was undoubtedly of animal origin.

In my opinion the danger lies in the jelly filling of the pies and in the manufacture of pies on the same premises where they slaughter. It will be

noticed in the Report that I examined some so-called "boiled" jelly. This specimen was warm when received and it was plated out at once. *B. coli* and other organisms developed after incubation. It is the custom to fill the pies with cooled jelly and one can imagine many ways for this jelly to become infected, and given warm weather or warm air as found in cook-houses, with the rate at which this type of bacteria grows, it is no wonder that these outbreaks are always cropping up.

To sum up the results of this work, the organisms under examination were very closely allied to *B. Gaertner* and can only be distinguished from *B. Gaertner* by the absorption test.

For purposes of reference I have named it *B. enteritidis* [Tank].

I wish to express my sincere thanks to Dr W. G. Savage and Dr Bruce White for their kindness and trouble in checking these results.

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INSTITUTIONAL HOOKWORM DISEASE IN A NON-ENDEMIC REGION¹.

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(With 1 Text-figure.)

CONDITIONS in institutions for children or defectives may become peculiarly favourable to the propagation of hookworm disease. The subject of the present study is a long-standing infection with *Ancylostoma duodenale* in the Hospital for the Insane at Goodna, Queensland, the largest of the three hospitals for the insane maintained by the State Government. The investigation was of special interest because the institution is in a region in which the general population is free from hookworm disease, owing to low rainfall, and because the infection was with *A. duodenale*, while the predominating hookworm infection of Queensland is with *Necator americanus*.

HISTORY OF THE INFECTION OF GOODNA HOSPITAL.

It was at the Hospital for the Insane at Goodna, then known as the Woogaroo Lunatic Asylum, that the first authentic case of hookworm disease in Australia was recorded by Hogg in 1889². The diagnosis was made at the autopsy of a male patient who had been in the hospital for ten years and had died of severe anaemia accompanied by general dropsy. Thirty to forty living *A. duodenale* were found adhering to the walls of the duodenum and upper jejunum. The hospital records prior to the death of this patient give little evidence to show whether hookworm disease was present in the institution. One patient died of severe anaemia in 1885, but the record states that no worms were found in the duodenum.

The source of the original hookworm infection cannot at present be determined. The facts that the worms infesting the patients in 1889 were *A. duodenale* and that those now present are of the same species, while the predominant hookworm of Queensland is *N. americanus*, suggest that the infection has been maintained not by repeated introductions, but rather by the perpetuation in the series of inmates of a very early infection. The hookworms

¹ The work on which this paper is based was conducted with the support and under the auspices of the Commonwealth of Australia, the State of Queensland, and the International Health Board of the Rockefeller Foundation.

² Hogg, J. B. (i. 1889). A Case of Death from Anaemia due to *Ankylostomum duodenale*. *Austral. Med. Gaz.* 133-134.

may have been introduced originally by patients who had come from Europe or China, as *A. duodenale* occurs commonly in parts of both places.

Following Dr Hogg's case, diagnoses of ancylostomiasis have been made at various times at the hospital, but the number of cases recorded has been small, as only patients showing recognised symptoms of the disease were investigated prior to 1922. The treatment of only those patients showing obvious anaemia would not tend to control the infection, and therefore it is not surprising that it was carried from the former Woogaroo Asylum buildings on the flats by the Brisbane River to the buildings of the present Hospital for the Insane on the higher land.

In 1922 the examination of all the patients in the hospital was then undertaken by the staff of the Hookworm Campaign at the invitation of the Medical Superintendent, Dr H. Byam Ellerton, and with the cooperation of the hospital staff. The investigation began on May 15 and ended on October 31.

DESCRIPTION OF THE HOSPITAL.

The Hospital for the Insane is situated near the town of Goodna on the south bank of the Brisbane River fifteen miles west of Brisbane, in a region having an average annual rainfall of 36·8 inches. Experience in the hookworm survey of Australia, has shown that hookworm disease is usually absent from the Queensland white population, even from the barefooted children, in sections where the rainfall is below 40 inches.

The grounds of the hospital are spacious and attractive. The buildings are mostly of brick, but a few of the old wooden wards remain. All of the wards are kept scrupulously clean. Every ward or group of wards has its fenced-in yard for the patients. Most of the yards are large and well-grassed, but some are worn almost bare of vegetation. Soil pollution is rare and the number of patients who go barefoot is small except in the wards containing the less teachable insane, such as imbeciles and violent patients. In such wards stools could usually be found by searching, but the general appearance of the yards was always clean.

Some of the wards are provided with modern water-flushed closets while others still have earth closets with pans. Gradually the transition is being made from the pan system to the far more satisfactory disposal by sewerage to septic tanks. The equipment of the newer bath-rooms and wash-rooms includes enamelled baths and basins and tiled floors and walls. About two months before the investigation began the closets of Ward M 4, which showed the highest infection rate, were changed from the system of outdoor pan-closets to water-flushed closets inside the building, but easily accessible from the yard. Ward M 5, the male ward having the next highest rate of infection, was in a transition stage, with a temporary pan-closet in use while a modern water-closet was being installed. The other three wards where infection was high were still using pan-closets.

PREVALENCE OF HOOKWORMS AND OTHER INTESTINAL WORMS.

All of the patients of the institution were examined. The hookworm infection rate for the hospital as a whole was found to be 23·4 per cent., while the rates for the several wards ranged from 2·0 to 72·3 per cent. Next to the hookworm the most common intestinal worm was *Trichuris trichiura*, for which the infection rate was 13·3 per cent. for the whole institution. The infection rate for this parasite was highest in the wards where hookworm infection was heavy, and lowest where hookworm infection was low, suggesting that the general conditions favouring hookworm transmission also favoured infection with *T. trichiura*.

The infection with *Oxyuris vermicularis* (4·2 per cent.) was a little more extensive in the hospital than among the general white population outside institutions, and varied irregularly between the different wards. The actual number of persons infected with *O. vermicularis* is distinctly higher than the number discovered by the examination of stools for ova, as the ova of this parasite are not evenly distributed through the stool.

The ova of *O. incognita*¹ were found about as frequently as those of *O. vermicularis*, but the distribution between wards was wholly irregular. The only other intestinal worms discovered were *Strongyloides stercoralis* and *Hymenolepis nana*, each of which was found only once. *Ascaris lumbricoides* was not found.

HOOKWORM INFECTION AS RELATED TO THE SOURCE OF THE PATIENTS.

If a considerable part of the infection with hookworms was being brought in by the patients when admitted, a much higher proportion of the infected patients than of the non-infected should come from those areas of Australia in which hookworm disease is endemic.

As a hookworm infection survey of Australia had been completed it was possible to classify the patients according to whether or not they had come from areas of endemic hookworm disease. The results of examinations in four wards are shown in Table I. It will be noticed that the proportions of patients coming from infested and non-infested areas are practically the same

Table I. *Hookworm Infection in Relation to Source of Patients.*

Wards	Infected patients		Non-infected patients	
	From infested areas	From non-infested areas	From infested areas	From non-infested areas
M 4	31	68	12	26
M 5	12	18	12	22
F 1	12	16	5	14
F 2	12	28	15	38
Total	67	130	44	100

¹ Kofoid, C. A., and White, A. W. (22. ii. 1919). A New Nematode Infection of Man. *Journ. Amer. Med. Assoc.* 72, 567-569.

for the infected and non-infected groups in the two male wards included in the table and differ only moderately in the two female wards. These observations strengthen the evidence that the infections were nearly all contracted within the institution.

HOOKWORM INFECTION AS RELATED TO LENGTH OF STAY IN THE INSTITUTION.

If most of the hookworm infection was contracted within the institution, the infected patients should have had the longest average length of stay. On the other hand, if the infection was chiefly contracted before admission, many infections should have disappeared gradually as the worms died out, and the non-infected patients should have been in the institution longest. In Table II is shown the average length of stay for the patients in the five wards where infection was highest and in one ward where there was little infection (M 9). Except in one of these wards (M 5) the average length of stay for the infected patients was longer than for the non-infected.

Table II. *Hookworm Infection as Related to Average Length of Stay in Hospital.*

Ward	Infected patients Stay in months	Non-infected patients Stay in months
M 4	133	72
M 5	89	94
M 6	101	88
M 9*	226	125
F 1	77	74
F 2	96	49

* Slight infection with hookworm.

DISTRIBUTION OF HOOKWORM INFECTION ACCORDING TO WARDS.

The amount of hookworm infection was very irregularly distributed between wards. Five wards had over 20 per cent. of infected patients, while several had about 3 per cent.

Starting with the assumption that most of the hookworm infection took place in the five highly infested wards, the records were studied to find how many of the infected patients in the other wards had been in one of the highly infested wards within the past six years and might have contracted the infection there. After deducting from the total number of infected patients in the more lightly infected wards, the number of these who had been in the five highly infested wards, the remaining number gave infection rates represented by the black columns in Fig. 1.

It will be noticed that these modified rates for five wards, excluding the hospital ward, were as low as 2 per cent. The infection rates of the two admission wards, M 2 and F 7, were 1.6 and 2.0 per cent., which may be taken as approximately the amount of hookworm infection brought into the hospital by new patients.

From the evidence presented it would seem safe to conclude that most of the infections in the institution took place in five of the twenty wards, M 4, 5, and 6, and F 1 and 2.

RELATION OF MENTAL CONDITION TO HOOKWORM INFECTION.

The outstanding factor determining the infection rates in the various wards was found to be the type of mental impairment of the patients. The wards containing a considerable proportion of imbeciles, idiots, and epileptics, and those in which there were violent insane patients, had been spreading hookworm disease, while the others had been relatively free. The ward where infection was greatest, M 4, contained among its 137 inmates, about forty-seven imbeciles and idiots and about sixteen epileptics. In these groups were a number of children. Ward F 2 contained congenital imbeciles and idiots and also

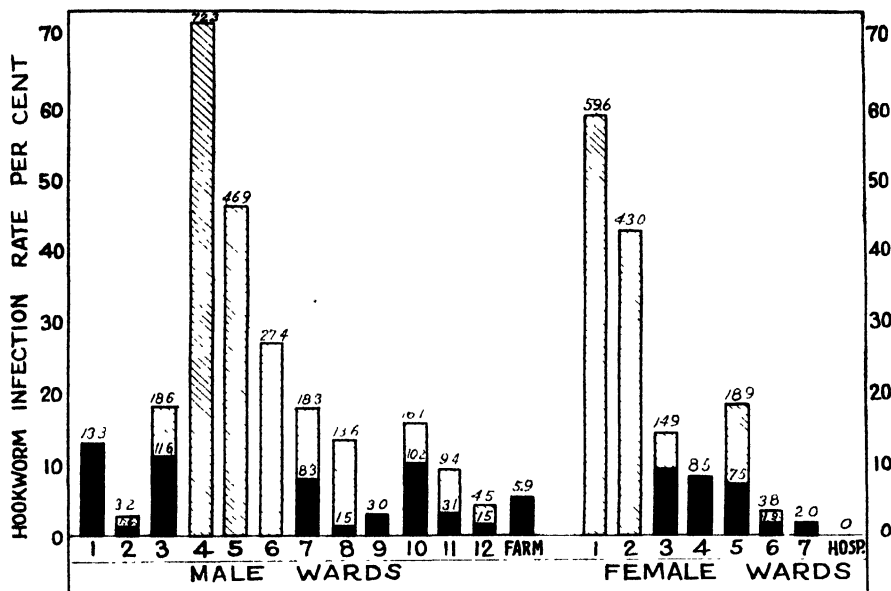


Fig. 1. Hookworm infestation rates by wards (total height of columns), and rates after excluding patients who had been in the five heavily infested wards within six years (dark columns).

noisy and troublesome female patients, including some of the epileptics. In this ward there were about thirteen idiots and imbeciles and five additional epileptics. There were a few children in the ward. Wards M 5 and F 1 contained the most violent adult insane, and Ward M 6 contained males having mental conditions similar to those of the patients in M 5 but much less violent. It will be noted that the infection rate in M 6 is the least of those of the five highly infested wards. Thus it appears that the wards containing the most unmanageable or unteachable insane were the ones in which hookworm disease had been spread most actively.

No evidence was obtained suggesting that the mental condition for which any of the patients were originally admitted was related to hookworm infection. The patients in the hospital were nearly all of the white race. Nine

persons were noticed who were of other races; and three of these were found to be infected.

INFECTION IN RELATION TO SHOE-WEARING.

According to nurses and attendants who had been long in the wards, 105 of the infected patients of the highly infested wards frequently went barefoot, while 121 usually wore boots. Of the non-infected patients of these wards only 29 had been habitually going barefoot, while 192 wore boots almost constantly. Many of the patients recorded as wearing boots took them off occasionally in the yards and may have been infected through the skin of their feet.

ANAEMIA DUE TO HOOKWORM INFECTION.

As the degree of anaemia accompanying hookworm infection is probably our best index of the damage being done by the disease, particularly where malaria is absent, as at Goodna, the percentage of the normal amount of haemoglobin in the blood was measured by the Tallquist scale for all infected patients and for the non-infected patients in the five heavily infested wards (M 4, 5, 6, and F 1, 2) and also in one of the least infested (M 9).

The results indicated that the average infection at the Hospital for the Insane was light, for the average loss of haemoglobin was only 2·2 per cent. of the normal in the five wards where the rate of infection was high. On the other hand, a small number of the patients were severely affected. All but one of the 22 patients having a haemoglobin percentage below 70 were infected with hookworms and were regarded as definite cases of hookworm disease. Seven patients, all of them infected, had severe anaemia; their haemoglobin percentages ranged from 40 to 55 per cent. of the normal. The average haemoglobin percentages for all infected patients was 78·4 in the male wards and 73·9 in the female wards.

The blood of ten of the more heavily infected patients showed a marked eosinophilia: 11 to 34 per cent. (average was 18·8 per cent.).

TREATMENT FOR HOOKWORM INFECTION.

The infected patients were treated either with oil of chenopodium or with carbon tetrachloride. Many persons received both drugs in the course of the series of treatments which proved necessary before a cure could be attained. The administration of treatments began in the male wards. At first the dosage for adults was either 1·5 c.c. of oil of chenopodium in a single dose followed in one hour by a purge of magnesium sulphate, and repeated in seven days; or a single dose of 3 c.c. of carbon tetrachloride followed usually, but not always, in four hours by a purge of magnesium sulphate. For those under seventeen years of age the dosage of chenopodium was 0·06 c.c. for each year, and the dosage of carbon tetrachloride was 0·06 c.c. for each cubic centimetre of the adult dose. In no case was there preliminary fasting or purging. The treatments were administered at 6.30 a.m. The patients treated were given no

breakfast, but all had their midday meal. Re-examinations were made not less than three weeks after the end of the treatment.

The reports from other countries regarding the effectiveness and safety of carbon tetrachloride, when used experimentally in animals or in thousands of patients infected with *Necator americanus*¹ led us to hope that a single dose of 3 c.c. would remove most of the hookworms present, even those of the species *Ancylostoma duodenale*, and would cure a large proportion of the patients. We were sadly disappointed, however, for only 23.1 per cent. of the patients who received carbon tetrachloride in this amount were cured, while 46.7 per cent., or twice as many, of those receiving the standard course of two treatments with 1.5 c.c. of oil of chenopodium were freed from infection. The counting of worms passed after treatment confirmed the relative failure of carbon tetrachloride in doses of 3 c.c. In subsequent treatments the dosage was gradually raised to 5, 6, 8, or 10 c.c. for the healthy adult. With 5 c.c. and 6 c.c. dosages the results were still slightly inferior to those obtained with the standard chenopodium treatment, but the efficiency of the 8 c.c. and 10 c.c. doses definitely exceeded that of two 1.5 c.c. treatments of chenopodium and was a little greater than that of a single undivided dose of 2 c.c. of chenopodium.

On account of the change in the method of treatment from time to time, the detailed results cannot all be brought together in a simple table. In Table III, however, are shown all the first treatments given and the percentage of resultant cures, and in Table IV are shown all subsequent treatments grouped together

Table III. *Results of First Treatments.*

Drug	Dose	Male wards		Female wards		All wards		%
		Treated	Cured	Treated	Cured	Treated	Cured	
Carbon tetrachloride	3 c.c.	91	21	10	1	101	22	21.8
	5 c.c.	0	—	20	6	20	6	30.0
	6 c.c.	0	—	20	5	20	5	25.0
	8 c.c.	0	—	21	14	21	14	66.7
Oil of chenopodium	1.5 c.c.	150	70	0	—	150	70	46.7
	repeated in 7 days							
	2.0 c.c.	0	—	15	11	15	11	73.3
Total		241	91	86	37	327*	128	39.2

* Excluding 15 first treatments which were of selected cases or otherwise irregular.

¹ Lambert, S. M. (1. vi. 1922). *Ankylostomiasis: Observations on Twenty Thousand Treatments with Carbon Tetrachloride*; Government Printer, Suva, Fiji.

Nicholls, L. and Hampton, G. G. (1. vii. 1922). Treatment of Human Hookworm Infection with Carbon Tetrachloride. *Brit. Med. Journ.* ii. 8-11.

Hall, M. C. (19. xi. 1921). The Use of Carbon Tetrachlorid for the Removal of Hookworms. *Journ. Amer. Med. Assoc.* 77, 1641-1643.

Lake, G. C. (12. v. 1922). Carbon Tetrachloride. A Drug Proposed for the Removal of Hookworms, with Special Reference to its Toxicity for Monkeys when Given by Stomach Tube in Repeated Doses. *U.S. Pub. Health Reports*, 37, 1123-1126.

Leach, C. M. (10. vi. 1922). Carbon Tetrachlorid in the Treatment of Hookworm Disease. *Journ. Amer. Med. Assoc.* 78, 1789-1790.

according to drug and dose regardless of the number of kinds of previous treatments. The percentages of cures are also shown for purposes of comparison.

Table IV. *Results of All Treatments after the First.*

Drug	Dose	Male wards		Female wards		All wards		%
		Treated	Cured	Treated	Cured	Treated	Cured	
Carbon tetrachloride	3 c.c.	8	0	1	1	9	1	11.1
	5 c.c.	2	1	0	—	2	1	—
	6 c.c.	19	9	0	—	19	9	47.4
	8 c.c.	14	11	8	7	22	18	81.8
	10 c.c.	121	93	45	33	166	126	75.9
Oil of chenopodium	1.5 c.c.	4	2	0	—	4	2	—
	2 c.c.	44	28	12	4	56	32	57.1
	2.5 c.c.	3	1	1	0	4	1	—
Total		215	145	67	45	282*	190	67.4

* Excluding 150 second chenopodium treatments in courses of two chenopodium treatments, shown in Table V, and 11 treatments not adaptable to this table.

SYMPTOMS AFTER TREATMENT.

Watch was kept for any symptoms which would suggest that the limit of tolerance of the vermifuge was being reached. On account of the mental condition of the patients minor symptoms were doubtless overlooked. The outstanding symptoms were vomiting and drowsiness. Vomiting occurred occasionally with both of the vermifuges, the frequency tending to rise gradually as the dose increased. The amount of vomiting was about the same after oil of chenopodium as after carbon tetrachloride, comparing separately the smaller and the larger doses of each and this symptom occurred most frequently immediately after the purge was administered.

Only one patient showed the persistent vomiting which occurs occasionally after the administration of carbon tetrachloride. A woman who had received 10 c.c. of the drug vomited two hours later, before the purge was administered, and vomited occasionally until the evening of the third day. She had had no previous treatment with carbon tetrachloride or oil of chenopodium.

Drowsiness was noted in a few cases after treatment with oil of chenopodium, but it occurred most frequently in the patients who had received 10 c.c. of carbon tetrachloride. It was observed twenty-eight times in the group of 169 treatments with this dosage, and usually came on soon after treatment, but disappeared a few hours later.

Six patients, three of whom had received 1.5 c.c. of oil of chenopodium and three 10 c.c. of carbon tetrachloride, were noticed to be cold and perspiring from one to three hours after treatment. One of those receiving carbon tetrachloride had also a rapid and feeble pulse. His earlier treatments had been with chenopodium only. These symptoms disappeared during the day.

The symptoms following the larger doses of carbon tetrachloride seemed to occur about as often in those who had not had previous doses of the same drug as in those who had.

HOOKWORM LARVAE IN THE SOIL.

The source from which the patients received hookworm larvae was of great interest, as the institution lies outside the hookworm belt. Soil sampling was accordingly undertaken in the hospital yards in the autumn on May 22, 1922, and was continued until the spring, ending on September 26, 1922. The wards receiving most attention were those most highly infested, Wards M 4, 5, and 6 and F 1 and 2. In all, 66 soil specimens were examined. The chances of finding large numbers of hookworm larvae in the earth of the yards were diminished by two factors: the cool weather of the autumn, winter, and spring, during which the investigation was carried on, and the fact that patients had been treated until their faeces were free from hookworm ova.

Soil samples of about 500 c.c. each were collected in glass bottles and conveyed to the Central Office of the Hookworm Campaign in Brisbane. There they were examined by the Baermann method as modified by Cort and his associates¹.

Each sample was put in a cylindrical sieve lined with cloth and placed in a large glass funnel closed at the outlet by a piece of rubber tubing and a pinch-cock. Water at a temperature of 46° C. was added until it rose above the bottom of the soil. The apparatus was then allowed to stand over night. The following morning the fluid at the bottom of the funnel was drawn off and examined under the low power of the microscope. Hookworm larvae and free-living nematodes were noted, and the former were counted. The sheathed and unsheathed hookworm larvae were recorded separately. Fluid was drawn from the apparatus and examined on three successive days, but most of the larvae were found in the first specimen.

In the examination of samples taken during the early part of the winter it was found that hookworm larvae were present in moderate numbers in a few samples, but only in the superficial layers of soil directly under recent stools. Many specimens taken at various depths where pollution was less recent showed no hookworm larvae, or exceedingly few. The evidence seems to indicate that in the season which is less favourable to hookworm larvae on account of lowered temperature and lessened rainfall, larvae may still hatch and develop, but will do so in considerable numbers only in the immediate vicinity of very recent stools. There had been rains shortly before the more heavily infected specimens were taken, and this doubtless favoured the development of the larvae. Although the number of observations were few, they seem to justify the conclusion that most of the infections of patients at Goodna took place outside the winter months, and that the larvae largely disappeared from the soil when conditions of temperature and moisture became unfavourable.

In Ward M 4, with the high infection rate of 72.3 per cent., only one place

¹ Cort, W. W., Ackert, J. E., Augustine, D. L., Payne, F. K. (i. 1922). The Description of an Apparatus for Isolating Infective Hookworm Larvae from Soil. *Amer. Journ. Hyg.* II, 1-16.

in the yard (Spot 7) was found at which the number of larvae was sufficient to indicate a possible source of infection. At the time of first and second examinations there were remnants of stools near this spot. In the first half of Table V the results of the examinations of specimens at and near the spot are shown. There was no shade in this place and the grass was thin and short from wear. The ground sloped gently. The surface soil was a dark garden earth, varying from dry to moist according to the rainfall of the previous fortnight. Six to twelve inches below the surface the soil was mostly yellow sand; at greater depths we encountered a compact mixture of sand and clay. In the north-west corner of the yard, where the ground was moister, the grass deeper, and pollution more common than elsewhere, no larvae were found in repeated samplings. From a hole in the asphalt walk in the yard some faeces and rubbish were taken which were swarming with free-living forms of *Strongyloides stercoralis*.

Results of examinations at Spot 42 in Ward F 2 which were also of special interest are shown in the latter half of Table V. The place at which the samples were taken was on a steep slope devoid of shade and covered by long grass; it was of made ground and consisted of garden earth, stones, and sand. At a depth of about 20 inches clay was reached. On the day the first specimen was secured an old stool was scraped away from the place. This may account for the large number of larvae found in the first sample.

In the north-east corner of the yard of Ward F 2 eleven specimens were taken at various depths on June 13 and 16 and September 26. Here the grass was thin and worn, and there was no shade except from buildings during a small part of the day. Pollution of the ground was frequent, and at one time there was an old stool on the surface at the place of sampling. Ova of the appearance of those of the hookworm were found in the sample from under this stool, but no hookworm larvae. The earth consisted of loam and stones, with clay 20 inches from the surface, and was moist or dry according to the previous rainfall. No hookworm larvae were found in any specimen from this locality.

In the yard of Ward M 5 one unsheathed hookworm larva was found at Spot 11 and one sheathed and one unsheathed larva at Spot 14. Both specimens were from the top six inches of soil. In the centre of the yard was a temporary latrine; the ground beneath it was wet from washings from above. Only one hookworm larva was found under the structure or near it. Possibly the use of disinfectants may have been an inhibiting factor.

Ward F 1, containing the violent insane women, ranked second in rate of hookworm infection. The yard is relatively small. It slopes rather steeply, and is absolutely devoid of vegetation. There is no shade except from fences and buildings. The slope is of hard, packed clay furrowed by rain, but toward the bottom of the incline a layer of loose sand has accumulated, and at this point stools were seen. A soil sample from the clay slope showed no larvae, and several samples taken at different depths at the bottom of the slope,

where the sand and pollution were noted, were devoid of larvae. The soil in the sandy spot consists of sandy earth, sand, and clay. At a depth of 18 inches or more the clay became very compact and was mixed with coarse sand and friable stone.

The conditions in this yard showed that vegetation and shade are not necessary for the development of hookworm larvae in considerable numbers. The absence of shade and vegetation may possibly have been offset by the increase of the moisture at the sandy foot of the slope occasioned by the run-off from the impervious clay surface above. Samples were taken in this ward to a depth of two feet, at which depth an impervious layer of clay and soft stone was reached, but no larvae were found. It seemed more probable that the larvae had died out than that they had descended to escape drying and cold.

Table V. *Hookworm Larvae Recovered from the Soil at Spot 7 in Ward M 4 and Spot 42 in Ward F 2.*

Locality	Date 1922	All depths		0-6 in		6-12 in		12-18 in		18-24 in	
		Sheathed	Un-sheathed	Sheathed	Un-sheathed	Sheathed	Un-sheathed	Sheathed	Un-sheathed	Sheathed	Un-sheathed
Ward M 4	May 22	27	2	27	2						
Spot 7	May 26	214	110	214*	110						
	June 2	43	1	0	1	43	0				
	June 20	8	3	7	3	1	0	0	0	0	0
	Sept. 26	0	0	0	0	0	0	0	0	0	0
Total at Spot 7		292	116	248	116	44	0	0	0	0	0
Ward F 2	June 13	412	6	412†	6						
Spot 42	June 16	11	0	11	0	0	0	0	0	0	0
	Sept. 26	0	0	0‡	0	—	—	0§	0		
Total at Spot 42		423	6	423	6	0	0	0	0	0	0

* In top 3 inches, 23 sheathed and 55 unsheathed.

† In top 3 inches.

‡ Top 12 inches in one specimen.

§ 12-24 inches in one specimen.

CONTROL MEASURES RECOMMENDED.

At the conclusion of the investigation at the Goodna hospital the following measures for the control of hookworm disease were recommended to the Superintendent of the hospital:

1. That the patients in the five wards where infection was high be examined for hookworm disease annually and treated with oil of chenopodium if infected. That all anaemic patients in the institution should be examined as soon as the condition is noticed, and given treatment if infected.

2. That patients be examined on admission and before discharge or transfer from the five wards of present high infection, and that those infected be treated, as far as practicable, to a cure.

3. That the yard of Ward F 1 be paved as soon as possible with asphalt

to prevent the development of hookworm larvae, and that the similar paving of Ward M 4 be considered after the results of the re-examination of the patients in 1923 are available.

4. That all patients be compelled to wear boots continuously while in the yards, locked boots being used when necessary.

5. That constant efforts be made to train the patients not to defecate on the ground, and to have stools removed from the yards as quickly as possible.

CONCLUSIONS.

1. An institutional infection with *Ancylostoma duodenale* has existed for over thirty-seven years in the State Hospital for the Insane at Goodna, Queensland.

2. The average severity of the infection is low, but a moderate number of heavy infections were present at the time of investigation, and deaths from hookworm disease have occurred.

3. The infection is essentially institutional; the hospital lies outside the hookworm belt, the residents in the vicinity are free from hookworms, and the predominating species of hookworms in Queensland is *Necator americanus*.

4. Most of the infection found in the twenty wards was contracted in the five which contained the highest proportion of unteachable and violent insane, and infection varied with the amount of soil pollution and the number of patients going barefoot.

5. Carbon tetrachloride given in doses of three cubic centimetres was found to be relatively inefficient in removing *Ancylostoma duodenale* and decidedly inferior to chenopodium in ordinary dosage.

6. Carbon tetrachloride in doses of eight to ten cubic centimetres had a distinct laxative effect. Few important symptoms were encountered after the administration of these large doses, but they are not recommended for general use.

7. In a subtropical region outside the belt in which there is sufficient rainfall to permit hookworm disease to spread in the normal white population, and in winter, when temperature and rainfall are lowest, hookworm larvae may hatch and develop in considerable numbers in the vicinity of recent stools moistened by showers, but the infestation tends to diminish rapidly and almost to disappear from the soil in a short time.

8. Hookworm infestation may be transmitted freely in the complete absence of vegetation if there is a high degree of soil pollution and sufficient moisture.

9. The effect of influences moderately unfavourable to hookworm larvae is to reduce the proportion of ova in the soil and thus limit the production of larvae developing to the infective stage.

EPIDEMIC ENTERITIS IN ABERDEEN DUE TO FOOD INFECTIONS.

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IN a preceding paper¹, details were given of an investigation which demonstrated that an epidemic of milk-borne enteritis occurring in Aberdeen in 1919 was due to infection of milk with dysentery bacilli of the Flexner type. It was further stated that the epidemiological and clinical features of antecedent epidemics of milk-borne diarrhoea were such as to suggest that the former epidemics were probably also dysenteric infections, although bacteriological investigation had failed to identify the causative organisms.

More recent experience of food poisoning outbreaks in Aberdeen indicates need for expanding the view thus expressed. Three succeeding outbreaks of enteritis have occurred in Aberdeen since the dysentery epidemic of 1919, and it has been found impossible to provide convincing bacteriological proof of the nature of the infection in any one of the three outbreaks.

It has to be noted, moreover, (a) that in all the infections cases were available for complete clinical and bacteriological investigation practically from their first onset; (b) that in their clinical manifestations the cases had the clinical features of either Gaertner infections or of infections of bacillary dysentery; and (c) that, notwithstanding the fact that all the resources of a modern bacteriological laboratory within the hospital were available and were utilised fully in the effort to arrive at a bacteriological diagnosis, the nature of the infections in the three outbreaks remains without exact determination.

In view of conclusions about to be set forth as a result of this experience, it appears desirable to record with some detail the enteritis investigations under review.

I. MEAT POISONING OUTBREAK.

During the forenoon of 5th November, 1921, a total of 30 cases of vomiting and diarrhoea occurred within a very limited area in the Footdee district of the city of Aberdeen. The police were early informed of the cases, and arranged for some of them being removed to the Royal Infirmary and to the Sick Children's Hospital. At the same time they communicated with the Health Department, and steps were immediately taken to investigate the outbreak. On inquiry, it soon became obvious that the cases were due to meat poisoning, and several of them were removed to the City Hospital for direct observation and treatment.

¹ Kinloch (VIII. 1923). *Journal of Hygiene*, xxi. 451.

Cases and symptoms. The inquiries made then and subsequently showed that there were 30 cases, of which 23 were females and seven males, and that all the cases were confined to 12 families. The ages of the sufferers varied from about 1½ years to 53 years—eight of them were under 5 years of age, seven were from 5 to 15 years, nine were from 15 to 30 years, and the remaining six were above 30.

The symptoms consisted of nausea, vomiting, diarrhoea and muscular cramps. In all the cases, except one where nausea alone occurred, there was vomiting. In 14 cases there was diarrhoea in addition to the vomiting, and in five cases there were cramps. In two of the cases with cramps there was no diarrhoea. In all the cases, symptoms developed within five hours of eating the suspected meat, and subsided within 48 hours. Apart from the nausea, vomiting was the first symptom in every case, and began in one case as early as half an hour after eating the suspected meat; in six cases it began in from one to two hours; in 12 cases in from two to three hours; in nine cases in from three to four hours; and in one case in from four to five hours.

In a few cases diarrhoea was reported to have occurred almost simultaneously with vomiting. In other cases it followed after a quarter of an hour to half an hour, or even an hour. In a small proportion of the cases, after the vomiting had apparently subsided, there was a recurrence of it as late as 10 or 12 hours after the initial symptoms, and in one case even as late as 36 hours. The stools were in most cases frequent and watery. There was no evidence of blood having been present in the stools. The cramps were mainly confined to the calf muscles and to the feet.

The temperature in almost all the cases was not above normal, although in at least one case it was 101° F. about nine hours after eating the suspected meat and about seven hours after vomiting and diarrhoea had begun. In this case there were cramps and a tendency to collapse. A moderate degree of collapse was noticed in several of the cases where vomiting and diarrhoea were pronounced.

The pulse was, in many of the cases, found to be considerably accelerated, being in a few cases about 130 to 135.

Severe headache was complained of in some cases, and in at least one case there was profuse sweating.

All the cases made a fairly rapid recovery, being practically restored to their normal health within 24 to 48 hours. In one case, which was removed to the *Royal Infirmary*, the patient was confined to bed for about four days.

The quantity of the suspected meat eaten by all the sufferers was small, and in several cases did not exceed half an ounce.

A cat in one house was said to have eaten some of the suspected meat and to have suffered also from vomiting and diarrhoea and to have died. On the other hand, a cat belonging to the vendor of the suspected meat was also said to have eaten a small portion of the meat without showing symptoms of illness.

Source of poisoning. Inquiry showed that all the persons suffering in the outbreak had partaken of tinned boiled beef supplied to them from a small grocery in the centre of the affected area, kept by a woman who had two unmarried daughters living with her of 19 and 23 years. The meat was taken from one tin containing about 6 lbs., which had been opened on the morning in which the meat was sold, and the contents transferred to a white enamelled metal plate. The plate was apparently clean, as also was the knife used for cutting the meat. It was admitted by the grocer that she had supplied portions of the meat to all the affected families, and that her own daughters had also partaken of it and had suffered. They formed part of the small group in which cramps were present as well as vomiting and diarrhoea, and one of them was the case in which illness began half an hour after eating the meat. The meat had, among all the affected persons, been eaten for breakfast.

The whole of the contents of the tin had not been sold by the time the police became aware of the outbreak of poisoning, and they accordingly took possession of the remaining 14 ounces of meat, and also of the empty tin, which, however, had been thrown into the ash bucket. The tin had been opened at both ends, and one of the ends was detached and not looked for by the police, and was not subsequently recovered.

The grocer, who had been in the habit of dealing regularly with tinned meat in her business, stated that the tin was not a "blown" tin, and a subsequent careful examination of the tin by the manager of a tinning factory did not reveal to him any evidence of the tin having been blown or punctured; but, as already stated, one end of the tin was awanting.

The grocer was certain that the meat showed no discoloration, and that it had no unusual odour, and so far as she knew, it had no unusual taste. In the case, however, of one or two of the families who suffered, it was stated that the meat had a peculiar sourish taste, and on that account a man in one of the houses, who had begun to eat the meat, spat it out.

So far as could be ascertained, no family that had received a portion of the meat from this tin wholly escaped symptoms of poisoning. Indeed, of all the 33 persons who partook of the meat, including the man referred to who said he spat it out, only three did not show poisoning symptoms.

The tin containing the meat looked comparatively fresh outwardly, and as if it had not been in stock for any great length of time. It bore a label which indicated that it was "*Fresh Boiled Beef*" packed by a well-known meat-production firm in South America.

The grocer stated that the tin was taken from one of two cases, each containing 12 tins, which had been received by her on 1st November from a wholesale merchant in Aberdeen. She also stated that she had previously sold in small quantities the contents of two tins from the same case, and that no complaint had been received from any of the purchasers. Inquiry among some of these customers by the Health Department confirmed her statement.

The wholesale merchant stated that the two cases of the tinned boiled beef

supplied to the grocer were part of a consignment of 14 such cases received by him on 3rd September from the London Company supplying the beef. All these cases had been sold to retail shops by the time that the outbreak took place—the whole of the shops, except one at Inverurie, being within the city of Aberdeen. Inquiries were made at every one of these shops as to whether complaints had been received of illness occurring in the families of persons to whom portions of this tinned beef had been sold, and in no case had any complaint been received, nor was any complaint received subsequent to the inquiry.

It would appear, therefore, that only one tin of the whole consignment was poisonous.

From the circumstances stated, it is scarcely open to doubt that the tinned boiled beef sold by the grocer on the morning of 5th November was the cause of the poisoning. It was the only meat or food sold by her that morning to the families involved in the outbreak that was likely to produce poisonous symptoms, and was, in any case, the only form of food sold by her to all the affected families. No other common food for these families was ascertained. Moreover, all the 33 persons who partook of the meat, except three, suffered.

Bacteriological and chemical investigation. Six samples of the suspected beef were submitted to examination at the City Hospital Laboratory. Five of these samples consisted of unconsumed portions of the beef that remained in five of the affected households, and the sixth sample consisted of the 14 ounces of beef that remained unsold from the suspected tin. Four samples of vomit, three samples of faeces, two samples of urine, and one sample of blood, all directly obtained from patients when their symptoms were most urgent, were also examined. The organs of the cat which died after eating the beef were also submitted to examination.

From all these materials cultures were made for the determination of the presence of *Salmonella* group bacilli, but with negative results. Anaerobic cultures from the same materials revealed no anaerobes of any significance in food poisoning.

Blood serum reactions from the patients in the hospital were also tested in reference to *Salmonella* type bacilli, the types used being those of the National Collection of Type Cultures in the Lister Institute. The results were negative.

Rats, mice, and guinea-pigs were fed with the beef, but showed no symptoms of illness; nor did certain rabbits and guinea-pigs when injected intravenously, intraperitoneally, and subcutaneously with a filtered emulsion made from the beef. The blood sera from these rabbits and guinea-pigs were tested for the presence of agglutinins, but without any positive result of diagnostic significance.

At the request of Dr Leighton, the Veterinary Officer of the Scottish Board of Health, samples of the meat were sent on 8th November to Mr Bruce White, Public Health Laboratory, University of Bristol, to be examined by him under the direction of Dr W. G. Savage.

Mr White reported that extensive cultural examinations of the meat for organisms of the *Salmonella* group had proved negative; but that the blood serum of a guinea-pig, which had received subcutaneously injections of filtered extract of the meat, developed a slight but definite agglutinative reaction in regard to the *Aertrycke* bacillus (Mutton type), thus indicating the presence of the toxins of this bacillus. The highest dilution, however, in which Mr White obtained evidence of definite agglutination, varied from 1 in 20 to 1 in 40, and it is obvious that such agglutinations are practically without any diagnostic significance whatsoever. The serum was tested against not only this bacillus, but also against *Bacillus aertrycke* (Newport), *B. enteritidis* and *B. paratyphosus* B.

At the request of Mr White and Dr Savage, samples of blood from four of the sufferers in the outbreak were sent to Bristol. The samples were taken about 26 days after the outbreak. Mr White found that an agglutinative reaction was obtainable with the *B. aertrycke* (Mutton type) with the serum in dilution of 1 in 10 from one of the patients. The sera from the three other patients gave a negative result.

Dr Savage, whose experience in the investigation of outbreaks of meat poisoning is greater than that of any other expert in this country, is of opinion that the outbreak under report was due to the toxins of *B. aertrycke* (Mutton type), but that no living organism of this bacillus, or of other meat-poisoning bacillus, were present—the bacilli having been destroyed in the process of canning.

The clinical characteristics of the outbreak and the brevity of the incubation period strongly support this view.

It remains to be added that a portion of the meat was examined chemically at the City Hospital Laboratory, immediately after the outbreak, for the presence of arsenic, antimony, lead, tin, and copper, and that no trace of these was found.

II. HOSPITAL ENTERITIS OF UNDETERMINED ORIGIN.

Following upon the Footdee Meat Poisoning outbreak, nine cases of which had received treatment in the City Hospital, and the last of which cases was discharged from hospital on 9th November, 1921, a limited epidemic of enteritis occurred among hospital patients beginning on 19th November, 1921. The treatment of the patients suffering from meat poisoning was so recent as to suggest at once that a carrier had been created in the hospital staff and was the cause of the fresh enteritis. Careful investigation, however, gave no support to this assumption. Not only so, but the clinical features of the cases suffering from meat poisoning as described were those of Gaertner enteritis, whereas in the hospital enteritis, as about to be described, the symptoms clinically were those commonly ascribed to bacillary dysentery.

Incidence. The outbreak originated on 19th November, 1921, and the cases—21 in number—were spread over an interval of eight days, the incidence on

successive days being 1, 8, 6, 0, 0, 3, 2 and 1. No age-period escaped, the youngest patient affected being an infant of nine months, while the oldest was 60 years of age. Sex appeared to be of no importance.

Distribution. The distribution of the cases is given in Table I, and reveals the interesting fact that the only ward which remained unaffected was the Ailing Infants' Ward, which contained for the most part debilitated infants whose powers of resistance might reasonably be assumed to be low. Another interesting feature was that only one member of the rather extensive staff was affected, the patient being a nurse who was on duty in a ward which furnished three of the more acute cases, and who developed the disease simultaneously with the ward cases. Table II, which gives the order of occurrence of the cases in the individual wards, suggests a more or less sporadic distribution.

Aetiology. Food contamination was suspected, but no evidence in support could be found. The same food, cooked in the same utensils, was supplied to the staff and patients indiscriminately, and no proprietary meat preparations were employed. A milk contamination was negatived by the fact that the Ailing Infants' Ward, which utilised a considerable amount of the hospital milk supply without further sterilisation, gave rise to no cases of infection. No history of recent diarrhoea or of dysentery could be elicited from any of the ward-maids, kitchen, nursing, or medical staff, while no case of diarrhoeal disease was being treated in the hospital at the time of the outbreak.

Symptoms. The symptomatology showed a remarkable uniformity in all the cases. The onset appeared to be acute, and in the majority of the cases could be definitely fixed in point of time. A period of nausea, culminating in vomiting, was followed in from two to three hours by severe diarrhoea. The temperature quickly rose to 101 or 102° F. and was accompanied by a much accelerated pulse rate. The stools were frequent, numbering in one case as many as 12 within the first 24 hours, and were characterised by the presence of mucus in 17 cases and blood in 11 cases. Some patients complained of headache at the onset. Rectal tenesmus was not noted, and there was no evidence of the occurrence of cramps. These symptoms subsided in from 12 to 72 hours, the tendency to diarrhoea being last to disappear. Recovery was complete in every case, and the only treatment employed was the routine administration of a purgative, usually castor oil, and the use of stimulants in severe cases.

Bacteriological investigation. Repeated examinations were made of the stools in every instance, and of vomited material whenever available. All these specimens gave negative results for the typhoid-dysentery group bacilli. Several blood cultures made in the acute stage of the illness were sterile. The blood serum of six of the patients in the second week of illness was tested against the following organisms, viz. *B. paratyphosus* B., *B. paratyphosus* B. (Mutton), *B. paratyphosus* B. (Newport), *B. enteritidis* Gaertner, *B. dysenteriae* V, W, X, Y, Z and Shiga. No agglutination was obtained in any diagnostic dilution in any of these tests.

Distribution of Cases.

Table I.

Pavilion	Inmates	Cases
Diphtheria	34	5
Scarlet Fever (A)	54	5
Scarlet Fever (B)	12	3
Reception House	9	3
Tuberculosis (Male)	49	2
Tuberculosis (Female)	69	2
Nurses' Home	100	1
Ailing Infants	17	—
	Total	21

Table II.

Pavilion	Serial numbers of the cases
Diphtheria	2, 3, 4, 6 and 10
Scarlet Fever (A)	9, 13, 15, 16 and 20
Scarlet Fever (B)	7, 8 and 9
Reception House	1, 18 and 21
Tuberculosis (Male)	11 and 12
Tuberculosis (Female)	14 and 17
Nurses' Home	5

Many similar epidemics of hospital diarrhoea have been reported formerly, and hospital authorities are indebted to Dr Harold Kerr, Medical Officer of Health, Newcastle-on-Tyne, for the following recent summary of such outbreaks in the Maternity Hospitals of this country:

Maternity Hospital, Newcastle-on-Tyne. Recurring enteritis experienced for some considerable time. Newcomers to the hospital were found to be especially affected.

Jessop Hospital, Sheffield. Similar outbreak experienced recently. Some serious defects in the drainage system were found, and these were remedied. No further cases have occurred since.

Maternity Hospital, Glasgow. Maternity Hospital opened in 1908, and three outbreaks have occurred during the last seven years. Only affects untrained midwives, and very rarely babies, patients, or trained nurses. Suggests that the diarrhoea is a neurosis probably dependent on change of food and discipline. Associated polyuria?

Queen Charlotte Hospital, London. Reported outbreak at this hospital some years ago. Due to the use of Perchloride of Mercury?

Union Maternity Hospital, Belfast. Three cases among nurses occurred in August, 1921. One student also affected. Was attributed to rhubarb. One other nurse was ill for a couple of days during the last week in October. These were the only cases reported during a period extending over 30 years.

Maternity Hospital, Edinburgh. No cases during recent years. Years ago some cases were reported, but after the hospital had been put into a sanitary condition there has been no recurrence.

Maternity Hospital, Leeds. Recently experienced a serious outbreak of gastro-enteritis among the babies, the latter being due to carelessness in dealing with infected napkins.

Rotunda Hospital, Dublin. None recently. Years ago trouble of this nature was occasioned through the water supply, found to be due to the water having been obtained from old contaminated tanks.

III. EPIDEMIC MILK-BORNE ENTERITIS IN ABERDEEN—JUNE, 1923.

In opportune fashion the Scottish Board of Health, having reference to the interest aroused by the tragic experience of botulism at Loch Maree, requested Local Authorities in a letter dated 13th October, 1922, to report forthwith to the Board all cases of food poisoning with a view to their fuller investigation. The response of the medical practitioners of Aberdeen to a circular letter from the Health Department indicating the Board's requirements in this connection has been of the best description. By means of this cordial co-operation, it was brought to the knowledge of the Health Department within a few hours of the appearance of the first of the cases that an outbreak of acute enteritis suspected to be due to food poisoning had occurred in Aberdeen on 6th June, 1923.

Milk infection. On first inquiry, it was ascertained that all the known cases had received milk from a retail shop of one of the largest dairies in the city. The milk supply of this dairy, amounting on an average to about 2000 gallons of milk per day, received from some 34 farms in the surrounding districts, is commonly pasteurised on delivery; but on inquiry it was ascertained that on 6th June the retail shop in question had run short of milk, and that ten gallons of a 20-gallon consignment of milk from a farm in the vicinity of Aberdeen had been retailed directly without pasteurisation. Concurrent investigation later elicited the fact that certain cases of enteritis typical of the cases in the outbreak under review had occurred in families which received milk from an entirely distinct source, namely, from a farmer who retailed milk by cart directly from his farm, and accordingly it appeared at first that so far as milk supply was concerned there could be no connection between the two groups of cases, although both groups of enteritis cases were determined to have exactly similar symptoms and approximately the same time of onset of illness. Further inquiry, however, elicited the fact that on 6th June the retailing farmer in the course of his distribution had run short of milk, and in order to supply his remaining customers had obtained one and a half gallons of milk from the premises of the dairy in question. Only those customers of the retailing farmer who received part of the one and a half gallons of milk from this source contracted the enteritis. It further appeared that the one and a half gallons of milk so obtained was taken from the receptacle from which raw milk was pumped to the pasteurising plant of the dairy, and which at the time contained in addition to other milk the remaining ten gallons of the 20-gallon consignment under suspicion. The remainder of this milk was subjected to pasteurisation in the usual fashion. There can be no reasonable doubt, therefore, that the 11½ gallons of milk which was retailed without pasteurisation was wholly

responsible for the infection. No other food was common to the infected households; and of the 43 families known to have received the infected milk, no family escaped infection, although 127 individuals escaped infection out of a total of 237 thus exposed. The ten gallons of infective milk in the dairy was exposed for sale about 10 a.m. on 6th June, and had been completely disposed of by 5 p.m. The additional one and a half gallons of unpasteurised and presumably infected milk was retailed between 8.30 a.m. and 9.30 a.m. on the same day.

Incidence and distribution. Of the total of 110 persons known to have contracted the enteritis, 14 were found to have sickened within 12 hours of consuming the infected milk, the shortest incubation period determined being five hours. An additional 47 persons were found to have sickened in from 12 to 24 hours after taking the milk; nine persons sickened in from 24 to 36 hours; and five persons were found to have sickened within from 36 to 48 hours; leaving 35 persons concerning whom the interval elapsing between the time of the consumption of the milk and the onset of illness was not determined. Of the 110 known cases of enteritis, eight sickened on the 6th June, 87 on the 7th, nine on the 8th, one on the 9th, and one on the 10th. The two latter cases were probably cases of contact infection, since original cases in these families occurred on the 6th or 7th June. In four cases the date of onset was not accurately determined.

As regards sex distribution, 47 of the 110 cases were males, and 63 were females, giving a proportion of 43 per cent. of males to 57 per cent. of females.

With reference to age distribution, four of the total cases were under 2 years of age; seven were in the 2 to 5 year age-period; 27 in the 5 to 15 year age-period; 21 in the 15 to 25 year age-period; 31 in the 25 to 45 year age-period; 17 in the 45 to 65 year age-period; and three above 65 years.

Source of infection. Inquiry and examination at the dairy supplying the milk revealed no evidence that the $11\frac{1}{2}$ gallons of milk causing the enteritis could have been infected on the dairy premises. Inquiry at the farm producing the 20-gallon consignment of milk from which the $11\frac{1}{2}$ gallons of infected milk was derived, elicited the fact that all the children of the farmer had suffered from an acute diarrhoea within a fortnight of the time the enteritis had appeared in the city. The farmer's wife and sister living with the children were engaged in the production of this milk, but since the dairyman had intimated to the farmer that his milk supply was under suspicion as a cause of the enteritis outbreak in the city, the reticence of the farmer was such that on inquiry no accurate information could be obtained of the nature or of the distribution of the diarrhoea in the farmer's family.

Symptomatology and treatment. The symptoms were remarkably uniform in the case under observation, and clinically were those attributable to bacillary dysentery infections, variation of clinical appearance having reference to intensity rather than to type. There was rise of temperature from 101 to 103° F. with pulse and respiratory rate in proportion. The onset was acute with obvious

shivering or sweating accompanied by abdominal pain localised mainly in the epigastric region. Vomiting, quickly followed by diarrhoea, developed in from one to four hours of the onset of illness, and the epigastric pain thereafter appeared to subside. Headache and backache were present in most cases, and within 24 hours the majority of the cases showed a considerable degree of collapse. Purging was intense in most instances, some patients having as many as 24 stools in the first 24 hours. Intermittent abdominal cramps were noted with occasional rectal tenesmus. Blood appeared in the stools of the vast majority of cases, but the most striking faecal feature was the amount of mucus, some of the stools in many of the cases being almost entirely composed of mucus. In the great majority of cases acute symptoms abated in from 36 to 48 hours. There were no deaths.

In four cases admitted to the City Fever Hospital polyvalent anti-dysenteric serum was given intravenously along with the oral administration of sodium sulphate. The remaining great majority of cases, however, received only aperient treatment and recovered with equal facility.

Opposed to the conclusion on clinical evidence that this enteritis had the essential features of a bacillary dysentery are the facts that out of an ascertained 110 cases none died, and that out of 106 of the 110 cases that received no specific therapy no case manifested the symptoms of chronic bacillary dysentery.

BACTERIOLOGICAL INVESTIGATION.

1. *Cases in Aberdeen.*

A. *Faeces from patients.* Thirty samples of faeces obtained from 21 patients at the height of illness gave negative results for non-lactose fermenting colonies of the paratyphoid-dysentery group. As it has been suggested by some British and American workers that streptococci may cause outbreaks of enteritis of this description, films were made from each sample of faeces. Streptococci were seen in some of the films, but in others none were found. Six samples of faeces were plated on blood-agar but only from two were streptococci obtained—*Streptococcus faecalis* and *S. viridans*.

Twenty strains of Gram-negative bacilli were isolated from McConkey plates and blood-agar plates. Some of these strains produced acid in lactose and in others sugars, without the formation of gas; others were non-lactose fermenters which did not conform to the sugar reactions of the paratyphoid group otherwise than in producing acid and gas in glucose and mannite and they did not react to any known serum. The sera of the four individuals in hospital gave negative results against the strains of organisms isolated from the various specimens of faeces.

B. *Blood cultures.* Three blood cultures were taken from patients in the acute stage of the disease. All were found to be sterile.

C. *Agglutination reactions.* Four samples of blood were taken in the second week from cases which had suffered severely from the enteritis. The sera were

tested against *B. paratyphosus* B., *B. paratyphosus* B. (Mutton), *B. paratyphosus* B. (Newport), *B. dysenteriae* (Flexner) V, W, X, Y, Z and *B. dysenteriae* Shiga. No agglutination was obtained in any diagnostic dilution even after incubation at 55° C. for 24 hours.

In view of the fact that in a previous dysenteric outbreak agglutinins had been found to be present in the blood serum of cases in the fourth week of illness although such agglutinins had not been demonstrated at an earlier stage of illness, blood serum specimens were obtained from six typical cases four weeks after they had suffered from the infection, but no agglutinative results of diagnostic significance were obtained in any of the cases. In general, these negative laboratory findings were confirmed by Dr Savage and Mr Bruce White working with similar laboratory specimens delayed in transit.

2. Cases at Farm.

A. *Agglutination reactions.* The sera of the five individuals at the farm who were capable of originating directly or indirectly the infection, were tested against paratyphoid, Gaertner, dysentery organisms. No agglutinations were obtained.

B. *Faeces.* The faeces of these five individuals, directly or indirectly capable of milk infection at the farm, were not found to contain any known pathogenic non-lactose fermenting organism.

The nature of the infection in this outbreak of milk poisoning remains, therefore, entirely undetermined, although the cases were subject to complete clinical and bacteriological investigation from their first onset.

Dr Savage is of opinion that this milk-borne enteritis was due to a living bacillus of unrecognised type of a strain allied to *B. dysenteriae*.

CONCLUSION.

In view of the Aberdeen experience, it seems reasonable to suggest, (*a*) that, even under the most advantageous conditions, modern bacteriological methods not infrequently fail to provide proof of the nature of Gaertner-dysenteric group infections, or (*b*) that viruses hitherto undetected can originate diarrhoeal outbreaks simulating clinically Gaertner-dysenteric infections.

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ON THE INHERITANCE OF ACQUIRED ANTIBODIES.

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THE presence of antibodies in the body fluids of the offspring of immunised parents has long been studied and variously explained. In 1912, Famulener² published a clear summary of the work upon the subject up to that date. Renewed interest in the problem has been aroused by the work of the Department of Animal Pathology of the Rockefeller Institute for Medical Research, where the transmission of immunity to the *Bacillus abortus* is being studied. It has been shown by Little and Orcutt³ that agglutinins for this bacillus are transferred exclusively by the colostrum, since a calf from which colostrum is withheld does not show this agglutinin in its serum, although a high titre of agglutinin may be demonstrable in both serum and colostrum of the dam. Later Orcutt and Howe⁴, using a method of separating the protein fractions of serum devised by the latter^{5,6}, showed that the appearance of agglutinins in the serum of a calf fed with colostrum of high agglutinin titre was closely related to the appearance of euglobulin, which is absent from the blood of the newly born calf. Further, Smith and Little⁷ showed that this absorption of agglutinin from the digestive tract also occurred when the calf was fed with serum rich in that antibody. The association of agglutinin with the globulin fraction of serum, and in particular with the euglobulin fraction, has been demonstrated by Orcutt and Howe⁴, who found that "the agglutinins are associated with the protein fractions which are precipitated up to and including 16.4 per cent. of sodium sulphate," and that "a large proportion of the agglutinins is associated with the fraction which is precipitated by 14.2 per cent. of sodium sulphate, euglobulin." Again, although a milk might show a high titre of agglutinin, with no corresponding increase in the proportion of its globulin, yet the agglutinin was removed from the milk by a concentration of sodium sulphate which removed the globulin fraction of the protein. In support of the direct absorption of protein from the digestive tract these

¹ This work was made possible by a grant from the Medical Research Council, to whom my best thanks are due.

² Famulener, L. W. (1912). *Journ. Infect. Dis.* x. 332.

³ Little, R. B. and Orcutt, M. L. (1922). *Journ. Exp. Med.* xxxv. 161.

⁴ Orcutt, M. L. and Howe, P. E. (1922). *Ibid.* xxxvi. 291.

⁵ Howe, P. E. (1921). *Journ. Biol. Chem.* xlix. 93.

⁶ *Ibid.* xlix. 115.

⁷ Smith, T. and Little, R. B. (1923). *Journ. Exper. Med.* xxxvii. 671.

authors quote the work of Langer¹, who prepared an antiserum to colostrum; using the precipitin test, he was unable to find substances in the blood of the new-born calf which would react with colostrum antiserum, whereas colostrum antiserum and cow blood did react. After suckling, and as early as six hours after suckling, substances appeared in the blood of the calf which reacted with colostrum antiserum. Raymann², whose work was done on goats, added evidence for the transmission of antibodies by the colostrum, by observing the serum content of normal agglutinin in the blood of kids before and after the ingestion of colostrum; he found that this body was transmitted to the kid in the colostrum of the mother, but that no quantitative rule was demonstrable.

More recently Lewis and Wells³ have extended the inquiry to man and find that new-born infants have little or no euglobulin in their blood; that euglobulins gradually appear; and that their amount is increased by the early ingestion of colostrum. Since it seems clearly established, at least in the case of animals whose young depend for their early nutrition upon the mother, that any existing maternal immunity for certain diseases is transmitted in association with the globulin fraction of the colostrum, it would be theoretically possible to increase this immunity by the administration to the infant of heterologous serum or colostrum of high titre in antibody for the particular disease. Lewis and Wells, however, sound a warning against such a practice, and point out that such protection "would be passive and therefore transient, while the sensitization would be active and permanent," and might lead to dangerous hypersensitivity in adult life.

The experimental work reported in this paper was done on the guinea-pig, whose young are little dependent on the female for their early food supply. It differs from the work quoted above in the use of the *B. typhosus* to create immunity in the parents, as opposed to the *B. abortus* which occurs naturally in the cow.

Method. The animals were actively immunised by the injection of regulated doses of the Standard Agglutinable Culture of *B. typhosus* prepared by the Standards Laboratory, University of Oxford.

Weekly doses of from 0.50 c.c. to 1.50 c.c. of the culture were given intraperitoneally, except in the case of females advanced in pregnancy, when they were subcutaneous; since it was found that the former method might induce abortion. The agglutinating power of the serum was determined with this culture according to Dreyer's⁴ procedure, blood being obtained from the marginal vein of the ear. The results are given in the form of the content of sera in arbitrary "Standard Agglutinin Units" (S.A.U.): this figure is arrived at by dividing the "titre" by a factor provided for each batch of Agglutinable Culture.

¹ Langer, J. (1907). *Verhandl. Ges. Kinderheilk.* xxiv. 70.

² Raymann, G. C. (1920). *Journ. Immunol.* v. 227.

³ Lewis, J. H. and Wells, H. G. (1922). *Journ. Amer. Med. Assoc.* lxxviii. 863.

⁴ *Med. Res. Council* (1920). Special Report Series, No. 51.

The animals were mated thus:

Table I. *Matings employed.*

Group	Male	Female
1	Not immunised	Not immunised
2	Immunised	Not immunised
3	Not immunised	Immunised
4	Immunised	Immunised

DATA OF EXPERIMENTS.

GROUP 1. (*Neither parent immunised.*) Before immunisation, in none of the 21 animals used was agglutinin for *B. typhosus* demonstrable in a dilution of serum of 1 in 25; similarly, the blood of the three offspring of the normal pair showed no agglutinin.

GROUP 2. (*Male immunised, female not immunised.*) The serum of the male showed a titre of 590 S.A.U. At the birth of the young, the maternal serum showed no agglutinin in a dilution of 1 in 25, nor did the sera of the three offspring, tested immediately, and at intervals of 24 and 48 hours after birth.

This result agrees with that of the majority of workers, and with Ehrlich's¹ original finding, that the male cannot transmit acquired immunity.

GROUP 3. (*Male not immunised, female immunised.*)

Case 1. At mating the serum of the female contained 280 S.A.U., but towards the end of gestation the titre had fallen to 110 S.A.U. Twelve hours after an intraperitoneal dose of 1.00 c.c. of suspension two young were born, one alive and one dead. Serum was obtained from the former 36 hours after birth, and its stomach content removed.

Titre of serum of mother = 110 S.A.U.	} Tests in duplicate.
Titre of serum of young = 290 S.A.U.	

The curdled colostrum from the stomach was extracted by grinding with sand and 5.00 c.c. of normal saline solution, spinning, and filtering. The clear fluid which resulted was substituted for serum in a Dreyer series, with suitable controls. No agglutination was demonstrable.

The higher titre of the serum of the offspring might be explained by the ingestion of colostrum rich in agglutinin, and failure to obtain evidence of antibody in the stomach content does not exclude this. In the first place I omitted to adjust the reaction of the colostrum extract; and secondly, Bond² has shown that grinding decreases the haemagglutinin content of the supernatant portion of human milk, although this fractional grinding process may increase the agglutinating capacity of an immune serum for its specific organism. Possibly Bond's findings may have a bearing upon such a result as the above.

Case 2. The female received three doses of 1.00 c.c. suspension, the last a fortnight before term. The two young were killed 18 and 36 hours after

¹ Ehrlich, P. (1892). *Zeitschr. f. Hyg.* **xii**. 183.

² Bond, C. J. (14. vi. 1919). *Brit. Med. Journ.* **i**.

birth, and blood samples and the whole content of the stomachs were obtained.

Titre of serum of mother = 500 + S.A.U.

Titre of serum of young (1) = 500 S.A.U.

Titre of serum of young (2) = 500 S.A.U.

The gastric content was treated as in Case 1, with the further precaution that the p_H of the extract was adjusted to that of guinea-pig serum by neutralisation with $N/100$ sodium hydroxide solution, the process being carried through at once to prevent further action of the gastric juice.

In neither case was any evidence of agglutinin in the colostrum extract obtained. No other food save bran was available for the young, and no bran was recognised in the stomachs.

In order to obtain serum from the young before there was any possibility of their receiving colostrum, they were removed from the tubes of the female near the end of pregnancy. (Under ether anaesthesia a free median incision exposes both tubes. The ovarian arteries are easily identified and clamped, and the tubes opened. Each set of umbilical vessels is then clamped, divided on the placental side, and the foetus withdrawn. After removal of the amnion, and drying to prevent contamination with amniotic fluid, blood may be obtained from the umbilical vessels. If the mother is to be kept alive, the operation must be done under aseptic precautions. The ovarian artery is now included with the stump of the tube in a silk ligature, and the remainder of the tube removed. Catgut is used to suture peritoneum and muscles in one layer, and silk for the skin stitch. This was done in two cases, the animals being afterwards convenient sources of a powerful agglutinating serum; in both recovery was rapid and uneventful.)

Case 3. The female had been immunised before mating, and during gestation received three doses of 1.00 c.c. suspension, the last 48 hours before operation.

Titre of serum of mother = 590 S.A.U.

Titre of serum of (three) young = 590 S.A.U.

Case 4. The female had been immunised before mating, and received a dose of 1.00 c.c. suspension intraperitoneally, six hours before operation.

Titre of serum of mother = 290 S.A.U.

Titre of serum of (two) young = 290 S.A.U.

Case 5. The female was immunised during the last month of gestation, and received a dose of 1.00 c.c. suspension, six hours before operation.

Titre of serum of mother = 30 S.A.U.

Titre of serum of (three) young = 18 S.A.U.

Case 6. The female had received three weekly doses of 1.00 c.c. suspension, the last six hours before operation.

Titre of serum of mother = 120 S.A.U.

Titre of serum of (three) young = 25 S.A.U.

Case 7. A subcutaneous dose of guinea-pig serum having a titre of 1 : 3200 was administered to a female advanced in pregnancy, and previously proved to have no typhoid agglutinin in her serum. The young were born 36 hours later, and samples of maternal and foetal blood obtained before the ingestion of colostrum could occur.

Titre of serum of mother 1 : 250 (25 S.A.U.)

Titre of sera of (two) young = each 1 : 25 (2.5 S.A.U.)

Case 8. (Control.) A single dose of 1.00 c.c. suspension was given to the female six hours before operation.

Titre of serum of mother = Nil.

Titre of serum of (one) young = Nil.

GROUP 4. (*Both parents immunised.*)

Case 9. Both parents received ten weekly doses of 1.00 c.c. suspension, the last four days before term. Blood was obtained from the (single) young 30 minutes after birth.

Titre of serum of mother = 590 S.A.U.

Titre of serum of young = 310 S.A.U.

Table II. *Relative amount of agglutinin in maternal and foetal sera of the cases reported.*

Case	Ingestion of colostrum	S.A.U. serum of mother	S.A.U. serum of young
1	Yes	110	290
2	Yes	500 +	500
3	No	590 +	590
4	No	290	290
5	No	30	18
6	No	120	25
7	No	25	2.5
8	No	—	—
9	No	590	310

Type of foetal immunity. In Cases 3, 4, 5 and 6 an attempt was made to demonstrate antigen in the foetal blood, the samples being taken 48 hours (Case 3) and six hours (Cases 4, 5 and 6) after its administration to the pregnant animal. Complement fixation tests were carried out with both maternal and foetal sera, as in the following typical experiment.

Minimum haemolytic dose of complement (Guinea-pig) = 0.0125 c.c.

Tubes	1	2	3	4	5	6
Normal saline (c.c.)	0.30	0.30	0.30	0.30	0.30	0.30
Complement (M.H. doses)	1	2	3	5	7	10
Foetal serum, heated for $\frac{1}{2}$ hr. at 57° C. (c.c.)	0.05	0.05	0.05	0.05	0.05	0.05
			1½ hours at 37° C.			
Sensitised ox R.B.C. (c.c.)	0.50	0.50	0.50	0.50	0.50	0.50
			1½ hours at 37° C.			
Haemolysis	Nearly complete	Complete	Complete	Complete	Complete	Complete

In no case was there fixation of complement in either foetal or maternal sera. There are, of course, several possible interpretations of this result; it does not necessarily preclude the presence of antigen in the sera tested.

Agglutinin in the organs. In each case an attempt was made to find out if the amount of agglutinin in the foetal organs varied with the particular organ.

Weighed portions of liver, kidney, and spleen were ground up with sand, in a volume of normal saline solution which was constant for each experiment. The resulting extracts were repeatedly centrifugalised and filtered until clear, and were then used in place of serum in Dreyer series. Similar organ extracts were also prepared from tissue washed in running tap water for 24 hours.

It was hoped that quantitative readings on a basis of weight might be obtained, but the results were very conflicting and unsatisfactory. Dilution for purposes of the test sometimes caused a precipitate in the original extract, for example of kidney; and the temperatures of both incubator (37°C.) and water bath (57°C.) caused precipitation of protein substances in the completed preparations. As an example of the inconstant behaviour of these extracts, an experiment may be cited in which a proportion of liver extract was added to the series of both agglutinating and non-agglutinating sera along with emulsion of *B. typhosus*. Precipitation did not occur in the latter series, although it occurred in the former, and also in controls of extract and saline only. Under such circumstances it was decided that the figures obtained were of no value for comparative purposes.

DISCUSSION.

It is clear that, as reported by other workers also, antibody may appear in the serum of the offspring of immunised guinea-pigs, without the previous ingestion of colostrum. On the other hand, in animals such as the cow, colostrum may be the sole source of certain at least of the antibodies found in the serum of the young calf. It is probable that the method of transference varies with the dependence of the young upon the dam for their early nourishment, and therefore different methods will be found to predominate in different species.

The origin of the foetal antibody in species independent of the ingestion of colostrum is still doubtful. It may be the result of a foetal reaction to antigen injected into the mother and reaching the foetus by the placenta; or there may be a transference of maternal antibody to the foetus by way of the placenta; or there may be a combination of both factors. It seems established (Case 7) that antibody introduced into the pregnant animal may reach the foetal circulation. With regard to the possibility that antigen molecules may pass through the chorionic villi there is little definite evidence; further, foetal tissues are so lacking in response to the introduction of antigen, that it is difficult to understand how they could show the amount of antibody found in some cases from the mechanism of active immunity alone.

The failure to establish a definite quantitative ratio between the titre of the maternal serum and that of the young—and this is the general experience

of those who have investigated the subject—would appear to exclude the transference being a physical phenomenon, for were this the case the amounts would be equal in both sera. It is generally accepted that the passage of the chorionic villi by fats and proteins is not a simple physical process such as diffusion, but possibly depends upon the presence of intracellular enzymes in their epithelium. Antigen bodies are of protein nature, and agglutinin is associated with the globulin fraction of the serum. It is therefore probable that transference, whether of antigen or antibody, is the result of cellular activity on the part of the chorionic epithelium. This explanation is not invalidated by any possible proteolytic activity of the intracellular enzymes; agglutinin, as previously stated, resists peptic digestion long enough for it to be absorbed directly from the stomach: indeed, Winterberg¹, many years ago showed that pepsin had the power of protecting—at least partially—typhoid agglutinin from destruction by hydrochloric acid, even in concentrations in which the acid is found in gastric juice.

The actual type of foetal immunity of this kind must remain for the present undetermined. Experimental observations, and especially those of a quantitative nature, will vary widely with the dependence of the young upon colostrum; with individual peculiarities in the reacting power of the animals; and with the behaviour of the antigens used.

CONCLUSIONS.

1. Agglutinin for *B. typhosus* is found in the serum of the young of immunised female guinea-pigs.
2. The agglutinin is present before the ingestion of colostrum.
3. Agglutinin as such may pass through the placenta.
4. The titre of the foetal may equal or exceed that of the maternal serum, but there is no definite quantitative relationship.
5. The complete mechanism of the production of this foetal agglutinin is unknown.

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¹ Winterberg, H. (1899). *Zeitschr. f. Hyg.* xxxii. 375.

THE SEROLOGICAL CLASSIFICATION OF *BACILLUS DIPHTHERIAE*

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HISTORICAL INTRODUCTION.

THE fact that diphtheria bacilli can be agglutinated by an anti-diphtherial serum has been known for many years.

Nicholas in 1896 demonstrated the agglutination of one strain of diphtheria bacillus by homologous antitoxic serum; and in 1898 (Nicholas, 1898 *a*, *b* and *c*) he found that by means of this serum, he could divide his strains of *B. diphtheriae* into two groups—an agglutinable and an inagglutinable. He showed, moreover, that agglutinability and virulence did not correspond, and that the same antitoxin protected animals against both groups of virulent *B. diphtheriae*.

Meanwhile, Nicolle (1898) attempted to make agglutinating sera in rabbits and white rats, using for injection the same strain as had been employed by Nicholas (Park 8, the American bacillus). From his negative results, he concluded that this particular strain did not produce agglutinins. In 1898, Bruno tested human sera for the presence of agglutinins. His sera were derived from cases of diphtheria and from healthy subjects, and he used an antigen made from a single strain of *B. diphtheriae*. His results persuaded him that a diagnostic test on these lines was of no value. In 1900, Lubowski published a paper on the diagnosis of diphtheria. He found that all his virulent strains fell into one serological group, whereas the avirulent strains and *B. Hoffmann* were not agglutinated by his sera. He laid stress on the difficulty caused by non-specific sedimentation of diphtherial emulsions and, to prevent this, he treated his antigens with glycerin.

In 1902, Wassermann reported his work on the use of precipitating sera for diagnosis of *B. diphtheriae*. He found that by this means he could differentiate this organism from *B. Hoffmann*. In the same year, Schwoner (1902) using a polyvalent horse serum, was able to differentiate *B. diphtheriae* from *B. Hoffmann*. He noted, in opposition to previous workers, that antitoxic serum did not agglutinate his diphtherial strains. It must, however, be remembered that the earlier antitoxin was made by injecting unfiltered broth cultures. Meanwhile, Mervyn Gordon (1901) attempted to differentiate *B. diphtheriae* and *B. Hoffmann* by means of specific guinea-pig plasma. He demonstrated definite serological differences between these two organisms, but finding to his surprise that all *B. diphtheriae* were not of the same type, concluded that as a means of identifying *B. diphtheriae* "the agglutination test promises to be of more use in its positive than in its negative aspects."

In 1903, Lipstein, continuing some earlier work, attempted to separate a few strains of *B. diphtheriae* into serological groups. He used rabbit sera, the animals being injected intraperitoneally with mixtures of virulent bacilli and antitoxin. He summed up his results by suggesting that all diphtheria bacilli had a common basal receptor mechanism, but differed in having in addition partial receptors peculiar to each strain. This far-reaching conclusion, though no doubt essentially true or very near the truth, was founded on the study of five strains, three of which appear to have been identical.

In the same year, Schick and Ersetting (1903) using serum prepared by Schwoner and others, confirmed its value in the differential diagnosis of *B. diphtheriae* and *B. Hoffmann*. The most interesting part of their paper deals with their description of the formation of "rough" and "smooth" colonies on agar by certain strains of *B. diphtheriae*. They extended the earlier work of Zupnik and showed that of the cases examined, 22 per cent. showed "rough" colonies only, 72 per cent. "smooth" only, while in 6 per cent. both types were found. They demonstrated, moreover, that the same antitoxin would protect against toxin, prepared from "rough" or "smooth" strains.

In 1912, V. Przewoski collected 50 strains of *B. diphtheriae* and five of *B. Hoffmann*. He made sera from two of the diphtherial strains and one Hoffmann. He found that the two diphtherial sera were identical and agglutinated all the strains of *B. diphtheriae* to 1/9600, but agglutinated the Hoffmann only to 1/150. The reverse held with the anti-Hoffmann serum. He used rabbits, injecting intravenously at first dead and then living cultures.

In 1914, Van Riemsdijk made univalent rabbit sera by injecting intravenously cultures killed at 60° C. He recognised different types of diphtheria bacilli, but, as he was only seeking a method of diagnosis from *B. Hoffmann*, he then made his sera polyvalent. He also noted the difficulty of spontaneous agglutination and advises prolonged heating of the suspension at 60° C.

In 1916, Langer made a univalent agglutinating serum by injecting rabbits with living diphtheria bacilli, suspended in antitoxin. He sums up his results by saying that there are two groups of *B. diphtheriae*, an agglutinable and an inagglutinable. Strains of the first group are all agglutinated by one type serum; the second group when injected into rabbits give rise to sera which do not agglutinate the strain used for injection but will agglutinate all the members of the first group. We can only suppose that he was dealing with one type to which both his groups belonged; and that the inagglutinability of certain strains was due to the unsuitable nature of the antigens employed (*vide infra*).

In 1919, Mason, working in an army laboratory, attempted to differentiate *B. diphtheriae* from organisms which resembled it morphologically. He made a monovalent rabbit serum by injecting heat-killed cultures of *B. diphtheriae*. He obtained a serum with a titre of 1/320, and found that 64 of 65 strains of *B. diphtheriae* were agglutinated at a serum dilution of 1/40 or higher. The diphtheroids tested did not agglutinate.

In 1920, Havens published the result of his study of 206 strains of virulent

diphtheria cultures, derived from cases, persistent carriers ("release cultures") and healthy carriers. His strains fell into two groups. His larger group (comprising 169) corresponds with that of earlier workers (Park 8).

In the same year, Durand (1918 and 1920) published papers on the serological types of *B. diphtheriae*. In an earlier paper in 1918, he had made horse and rabbit agglutinating sera and had shown that of 96 strains which he could emulsify, 70 fell into four groups. Later, he brought his total to 255 strains, 18 of which were non-emulsifiable. His horses were injected intravenously with living bacilli. He also tried the same process with goats, but the results were disappointing. He states that "les injections intraveineuses de bacilles diphthériques tués par la chaleur, sensibilisés ou vivants à doses progressives amènent le plus souvent la mort plus ou moins rapidement avec ou sans paralysie." He therefore used a mixture of antitoxin and bacilli for injection into these animals. He finally determined five types, which he found absolutely specific by the method of absorption. In a paper written in conjunction with Jean Guérin (1921) he investigated the relation of serological type to family incidence and epidemiology, and concludes that "the unity of type in an epidemic seems established." As these writers say, this may explain results such as those obtained by Langer. In a final paper, in 1921, Durand studied the fermentative reactions of 224 strains. He finds glucose and laevulose are always fermented; mannite, dulcitate, sorbite, xylose, mannose, lactose, starch inulin and glycogen never; glycerin, galactose, maltose, saccharose and dextrin variable.

His types I and II differ from the remainder in sugar reaction, and his type II ferments saccharose.

In a recent paper, Park (1922) and his colleagues who examined Durand's strain "corroborated fully" his findings as far as they relate to the serological type.

Early in 1922 a paper appeared by Bell on this subject. He examined 133 strains, three of which he was unable to emulsify. Of the remainder, 80 per cent. belonged to three types; 13 per cent. were included in type I, which was Havens' larger group and included Park 8, the strain which has been used for so much experimental work. His second type corresponds to Havens' smaller group and claims 6 per cent., while the remaining 61 per cent. were included in type III. 20 per cent. were left untyped. He used rabbit sera, having tried guinea-pigs without success; he gave intravenous injections at five-day intervals, using at first heat killed and later living bacilli together with antitoxin. The mortality among his animals was high. His agglutinations were carried out with suspensions of bacilli grown on pea flour agar; the mixtures of sera and bacillary suspensions were heated at 55° C. for four hours before reading.

This brief summary of the literature shows that most of the earlier work was carried out with a view to finding a serological means of distinguishing *B. diphtheriae* and *B. Hoffmann*, or between virulent and avirulent strains of

B. diphtheriae. In recent years, the distinction between *B. diphtheriae* and *B. Hoffmann* has ceased to be a matter of great difficulty. Moreover, as far as clinical medicine is concerned, a virulence test gives more valuable information than serological study. Thus we find that Durand and Guérin stress the epidemiological value of their work which, though commenced earlier than that of Havens and Bell, may be said to present the most modern aspect of the subject. The object with which a research is undertaken, though not influencing the facts elucidated in the course of that research, must alter the direction of the work, and the value attached to its different phases. And thus the earlier tendency to unify all types of *B. diphtheriae* in an effort to provide a basis for differential diagnosis is, later, replaced by a schismatic phase—a stressing of differences.

SCOPE OF THE WORK.

In the course of his research Major A. S. W. Bell very kindly grouped, by means of his type sera, several strains of virulent and avirulent *B. diphtheriae*, which we had in our collection. Certain of these strains, already classified by him, we chose for making sera. In this way our first three groups correspond with his (*vide infra*). From the strains which failed to react with the three-type sera originally used, haphazard selection was made for the preparation of univalent sera. In many cases several of the chosen strains proved to be identical, so that we were able to test the homogeneity of each group by means of cross agglutination and absorption experiments. In this communication we are dealing with 348 cultures, all virulent. Some of the strains were used in an earlier investigation on the value of the intracutaneous test (1921 and 1922) and their virulence checked by the subcutaneous method. The remainder have been tested by the intracutaneous method only. Sera have been made from 33 of these strains, 36 rabbits and four horses having been employed in their preparation. The number of individual experiments carried out prevents the presentation of complete protocols. We propose, therefore, to give full technical details of the methods employed but content ourselves with a summary of the results obtained.

TECHNIQUE.

(1) *Antigens*. We deliberately selected a medium in common use, and as Loeffler's serum was found to be suitable for every stage of our work, we used this throughout. The organisms were isolated on Loeffler's serum inspissated in Petri dishes. Antigens were made from 24 hour growths on the same medium. Small quantities, suitable for routine typing, were obtained from two slopes. For preparing large amounts we proceed as follows: the serum mixture is poured into a 100 c.c. flat-sided bottle. The bottle is then placed on its side in the oven, the serum inspissated by heating at 85° C. for one hour; the fluid which exudes is then poured off, and a further amount of serum mixture added. The bottle is then placed on the opposite side in the oven, and heated again

for one hour. The fluid is again poured off and the bottle incubated at 37° C. for one day to test its sterility. We have thus two opposing surfaces covered with Loeffler's medium. An overnight slope culture is emulsified in broth. This is poured into the bottle and carefully washed all over both prepared surfaces. The bottle is then placed upright in the incubator. The next day the fluid contents are carefully poured out down one of the bare sides of the bottle. Saline (0.9 per cent.) is then added and the growth emulsified. The emulsion is heated at 60° C. for 30 minutes and diluted to contain 10 thousand million or 2 thousand million organisms per c.c. (judged by an opacity standard). Phenol is added to a concentration of 0.5 per cent. The former "thick" antigen is used for absorption experiments, and for injection into horses. The "thin" antigen is used for agglutination and injection into rabbits. The "thick" antigen can be diluted with Phenol saline to make thin antigen as required. From each bottle nearly 20 c.c. of "thick" antigen can be obtained. Large amounts of "thin" antigen were generally made from two bottles, and can be kept in stock for serial experiments. Owing to the variability in agglutinating properties of antigens prepared from the same strain at different times, this is fortunate.

(2) *Sera*. In view of previous work on this subject, and for convenience, rabbits were selected as the animals to be used. At first we employed living avirulent cultures, being deterred from using virulent strains by the high mortality reported by previous workers (Durand, Bell, etc.) and thinking it unwise to complicate matters by injecting our animals with antitoxin. We soon found, however, that we could safely employ killed virulent strains and in this communication we shall only mention sera so made. Rabbits were injected intravenously with 1, 2 and 3 c.c. of "thin" antigen on consecutive days. After an interval of eight or ten days an experimental bleeding was taken; 3 c.c. were then injected, and after an interval of ten or twelve days another experimental bleeding; if the titre of the serum was 1/400 or higher the animal was bled twice with a day's interval between the bleedings; the animal was then rested for four or five days and its serum re-tested, subsequent procedure depending on the titre found. Of the 36 rabbits used only one died in the course of immunisation, and two others never produced serum of a higher titre than 1/100. The titres produced by the remainder may be tabulated as follows:

1/500	1	1/1000	4
1/600	4	1/1200	2
1/800	15	1/1600	7

The time needed for immunisation was generally very short. Serum was obtained from 14 rabbits in 16–21 days after the first injection; from 11 rabbits in 22–28 days after the first injection; from four rabbits in 29–35 days after the first injection; while four rabbits did not produce usable serum until the 54th–60th day. It will be noticed that on the whole the sera produced were of low titre. They were, however, extremely specific as can be seen from Tables

III and IV. For routine agglutination we employed them in a final dilution of 1/100, 1/200 and 1/400.

Our colleague Mr Buxton injected four horses with four separate strains. "Thick" antigens were employed. Three of the horses produced sera of a titre of 1/1500 or higher. One never reacted at all, though a rabbit injected with the same strain produced a serum of 1/800 titre. These sera are considered later.

(3) *Agglutination.* After trial had been made of different apparatus, temperature, etc. the following method was finally adopted. Small tubes, 7.5 x 0.5 centimetres are employed. 0.9 per cent. saline is employed for all dilutions. Each tube contains 0.5 c.c. diluted serum and an equal quantity of "thin" antigen. For routine work at least four sera are tested at one time against each antigen. We have found this forms a safer control than the inclusion of normal serum dilutions and saline in the test. The serum antigen mixtures are placed in a water bath at about 55° C. for one hour, the tubes being partially immersed after Topley's method. Readings are then made by naked eye, the agglutination being very definite.

(4) *Absorption.* To 2 c.c. of each antigen is added 0.1 c.c. of serum diluted according to its agglutinating titre. Thus, if the titre of the serum to be tested is 1/800, 0.4 c.c. of serum is diluted to 1 c.c. with saline. 0.1 c.c. of this (containing 0.04 c.c. of serum) dilution is added to each 2 c.c. of "thick" antigen, so that the final concentration of serum is 0.04 in 2.1 c.c. = 1/50.

The mixture is incubated at 37° C. for two hours, centrifuged (or allowed to sediment) and the supernatant fluid removed and tested for agglutination against the appropriate antigen in final dilutions of 1/100, 1/200, 1/400, 1/800. We have found recently that it is unnecessary to employ centrifugalisation. After two hours' incubation the racks containing the tubes are placed in cold water over-night. The next morning the supernatant fluid is sufficiently free of organisms to admit of being pipetted off and tested. This saves a great deal of time when 30 or more absorption tests are being made. We always include in the test at least four positive and negative controls. That is, sera mixed with their corresponding antigen and with saline. We make a preliminary test on one positive and one negative control. If the former shows that absorption is not quite complete, we re-incubate for one hour; if the absorption is very slight we add more antigen and re-incubate; if the negative control does not show agglutination in a sufficiently high dilution (three or four tubes completely positive in a cent. per cent. series), *i.e.* in a dilution eight or sixteen times the lowest of the series, the experiment is discarded and much time saved.

(5) *Difficulties.* As far as sera are concerned we have had little trouble. We are, however, convinced that they deteriorate rapidly even when kept in the cold room. They certainly compare very unfavourably, in this respect, with other agglutinating sera. Fortunately being so specific, they can be used in low dilutions. It is the antigens that have caused us most anxiety. In the

vast majority of cases the growth on a 24-hour Loeffler culture can be emulsified easily in 0.9 per cent. saline. Antigens made as described above remain suspended for many hours, and, when the organisms settle only need a thorough shaking before use. Certain cultures, however, yield antigens that are not completely homogeneous; sufficient material, however, remains in suspension for use in the test. In a very few instances (as is noted by Durand, Bell, etc.) the culture cannot be emulsified at all. Thus we find every stage—a perfect, a usable and a useless antigen. Some antigens again though apparently homogeneous, sediment when diluted with serum or saline and heated. This “non-specific” sedimentation can generally be distinguished from specific agglutination in three ways. In the first place, the aggregation of the organisms is much less compact than in the latter, in the second the sedimentation takes place in the higher rather than lower dilutions (the serum acting as a protective colloid), and lastly the reaction affects several, perhaps four or five sera. With some sera, however, this non-specific reaction is more marked than with others, so that unless several sera (three at least) are employed in each test erroneous conclusions may be drawn. It must be emphasised that controls of normal serum and saline are not of much practical value. They may show no agglutination, and yet non-specific reactions may occur with the same antigen and other sera. This tendency to non-specific reaction with serum and difficulty in emulsification seem to be due to physical causes, being different degrees of instability. We tried to overcome these difficulties by heating, reducing the salt content, the action of acid and alkali, etc. but with no success.

In the course of quite a different set of experiments, however, we found that glycerine in a final concentration of 1/16 completely inhibited the specific agglutination of a Mutton strain of *B. aertrycke*, and after trial of different strengths of glycerine we finally added one part of glycerine to 19 parts of diptherial antigen prepared in the usual way. The mixture was well shaken and heated at 55° C. in a water bath for eight to ten hours, being shaken repeatedly. By this means suitable antigens have been obtained from all except three of the unstable strains. Specific agglutination is not interfered with; the positive results obtained have been checked by absorption experiments. Antigens that sediment non-specifically do not absorb agglutinin, except from their homologous sera. The three strains, above-mentioned, failed to absorb the agglutinin from any of our sera and are included amongst the “unclassified” in Table I.

These phenomena may be partially explained by the presence of “rough” and “smooth” variants in the cultures. We have had more than one strain which, after providing suitable antigens for a long time, has gradually developed the tendency to clump naturally and we have watched one particular strain pass through every phase until it became impossible to emulsify it in the ordinary way, though glycerination and heating proved successful. At this stage it was plated and several colonies found to be all equally refractory.

These changes may also take place in old stock antigens; which can be restored to usefulness by glycerination and heat. It is interesting to note that we were able to produce an agglutinating serum in response to intravenous injection of one most refractory strain. The serum so made was tested later against the same emulsion after treatment with glycerine. It is the strain 668 in Tables III and IV, where it is seen to act quite specifically in direct agglutination and absorption.

Our other difficulty was of an entirely opposite character. Certain antigens do not agglutinate with the corresponding sera, and for this reason we always make it a rule to prepare a second antigen if the first one made from an unknown strain reacts with none of our type sera. In spite of this precaution we have twice prepared sera from cultures classified as "unknown" only to find that the sera so obtained were identical with one or other that we already possessed. Such antigens will absorb the agglutinins from their own type sera and can thus be classified (*vide infra*).

Nicholas (1900) records the fact that one strain originally inagglutinable became agglutinable after being subcultured in the laboratory over a period of a year. This inagglutinable tendency is more frequently met with in antigens from old laboratory cultures than recently isolated strains. We have every reason to believe that the relative agglutinability of these antigens depends upon the proportion of agglutinable individuals present, though we have no direct evidence on this point with regard to *B. diphtheriae*. We have, however, demonstrated it clearly for certain strains of *V. cholerae*.

SOURCE OF MATERIAL AND GENERAL RESULTS OBTAINED.

The 348 strains were derived from cases, contacts, convalescent carriers, and healthy carriers together with a few cultures of unknown origin transferred to us from other laboratories. The general results are shown in Tables I and II.

It will be seen that by direct agglutination we were able to classify in ten groups 323 strains—including 13 which showed agglutination with the sera of groups 3 and 4. Of the 25 unclassified by this means nine absorbed the agglutinins from one or other of the type sera. Unfortunately, only three of the 13 in groups 3 and 4 were preserved and tested by absorption; two were type III, one type IV. These are included in Table II, which gives details of the origin of the cultures. It will be seen that group 3 contains the largest number of strains; group 5 about half the number; group 2 is the third most important. Group 1 is probably over-represented as 14 of its members were of unknown origin and there are almost certainly several strains of Park 8, under different designations. In different series the relative preponderance of each group varies with the source of the material. Thus, even in large series of strains the influence of local epidemic conditions may obscure the vision of the worker who does not search far and wide in his attempt to achieve catholicity. Langer and Mason found one group while Havens' strains are represented by our groups 1 and 2. He missed entirely our largest collection. It follows that

it is of no value to work out the percentages of each type found in a miscellaneous collection such as ours, most of which were derived from the London area, some from Scotland and the provinces, and some from America.

What is important is to realise that organisms of every group can be cultivated from "cases" or carriers, and there is every reason to believe in the existence of still further groups which we have not discovered. Nor would there seem to be any evidence for the view that one type is responsible mainly for cases or exists chiefly in carriers. At first sight it might appear that types V and IX were specially associated with cases, III and II being relatively more frequent in the carrier. But as the carriers were mainly taken from an area in which a type III epidemic was raging, and were many of them more or less direct contacts, any such conclusion would be unjustified. In the same way it appeared at first sight as if types V and IX were specially associated with nasal infection. Of 14 nasal cases, six were associated with type V and four with type IX. Here again three of the latter occurred in one institution and four of the former in another, the cases being so mild in character that they could be regarded as "Bacteriological Diphtheria," *i.e.* carriers. We have examined only five cultures from the ear. Two belonged to type III, two to type V, while one, though showing affinities to other groups, was classified by absorption as type I (*vide* No. 502, Tables III and IV).

SEROLOGICAL RELATIONSHIPS.

If we examine Tables III and IV we find that, as far as direct agglutination is concerned we have ten absolutely specific type sera. When we turn to the absorption results we find a variable relationship between types III and IV. These were tested several times and sometimes partial cross-absorption was demonstrated, sometimes not. Otherwise the ten type sera are specific by this test also. The five "Intermediate" sera are of great interest:

502 by agglutination	type I (VII, VIII)	by absorption - type I
301	III (IV, V, J, VII)	III (1)
525	III, V, IV	IV
506	III, IV, VII	III
519, 46	II, VII	II, VII.

We have also three sera which react only with their homologous strain.

The "mongrel" sera will be seen to be mainly associated with groups 3 and 4, the strains chosen for producing the sera having been difficult to assign to one of these types (301, 525, 506). As will be seen from Table II this is the most important association with which we have met. Moreover, there is a slight association between these two type sera (*vide* Table IV). Now most of our strains have come from an area where type III organisms are epidemic. It is therefore suggestive that the "mongrel" strains should show a relationship with type III compatible with the theory that they are either ancestors or descendants of this strain. We must also expect to find in other areas strains more nearly related to types IX and X, etc. and to the unknown groups which may be found elsewhere. Table VI shows the relationship between the sero-

logical types of *B. diphtheriae* found by previous workers and ourselves. Bell's sera and our own show interesting differences. In each case type I was made by injection of the same strain. The difference in type specificity is curious and probably due to different methods of immunisation. Dr W. M. Scott kindly sent me some serum which agglutinated all our ten types though the strain with which it was prepared, was, when tested against our own sera, specific type III. The second point of special interest is the fact that Bell regarded his three sera, Andrews, Mulaney and Blackburn, as identical; our results are shown in the table.

AGGLUTINOGENESIS.

We have seen that, with our method of immunisation in the rabbit, we obtain from most strains highly specific sera, while a few strains give more catholic results. Presupposing that horse sera would be less specific, we decided to study the production of agglutinins in the horse in response to injections of types I, II and III. We hoped that the horse sera would gradually become non-specific, and that the relationship of the strain used to the strains of the other groups could be demonstrated by this lack of specificity.

The results obtained with the sera of three horses are shown in Table VI. *H* 1154, injected with type I, shows at first relationship with III, IV, VIII and IX; then with II, V and X.

H 1156 injected with type II was never found to be specific but showed relationship between II and I, III, V and IX, and later VIII, VII and VI but never with X.

H 1173, injected with type III, remained specific for a long time and then showed relationship between III and II and VIII, then I, IV, V, IX.

Now if we consider the strains 55 and 301 we find that the former produces an almost specific rabbit serum, and if injected into the horse, only after a long while shows a tendency to catholicism. 301, on the other hand, even in the rabbit, produces a serum with broad sympathies. 55 is more specialised, but both strains contain in common antigenic components = III, I, IV, V, VII (*vide* Tables III and IV). It is in the relative predominance of antigenic component III that they differ.

We may then assume as a working hypothesis that all diphtheria bacilli have the same qualitative structure, and that every strain contains antigen elements I, II, III, IV, V, etc. In group 1, element I predominates and sera can be produced which show group 1 agglutinins only. But the other elements are there and when sufficient of them have been injected the animal will respond. This latter suggestion is strongly supported by Table VI, which shows the agglutino-genesis in the three horses. It will be seen that the non-specific group reaction is a miniature of the specific and comes on later. This would accord with the hypothesis of quantitative variations but qualitative identity in composition as judged by agglutino-genetic power.

It may perhaps be objected that, if this qualitative identity exists, it could

be established by means of absorption tests. Thus, any diphtherial agglutinating serum could be absorbed by any one antigen provided enough of the antigen be employed. We have tried such an experiment, treating all the ten type sera with large amounts of antigen of one strain (15 times the amount usually employed). But no cross-absorption could be demonstrated. It may be that we could not use sufficient antigen; this, however, is unlikely as we should have found faint indications of absorption at any rate.

That the agglutino-genetic capacity of two antigens might be qualitatively similar even though they show no connection by agglutinating or absorption tests is easy to conceive; for in the former case the actual agglutino-gen may be formed from the antigen in the body of the animal injected.

VALUE OF SEROLOGICAL CLASSIFICATION OF *B. DIPHTHERIAE*.

We have seen that the previous work on this subject has been actuated by different motives. Our own object in undertaking this study was primarily concerned with an enquiry into the relationship between virulent and avirulent strains of *B. diphtheriae*. We hoped that, by studying their agglutinating properties we might find a clue to their relationship. We soon realised, however, that it was wiser to limit ourselves at first to the classification of virulent strains, and this for two reasons:

- (1) We had a sure test for the genuineness of our strains, *i.e.* a virulence test.
- (2) We found that, though we could make sera from avirulent strains and thereby classify virulent strains and *vice versa*, still the serological reactions of the avirulent organisms were much less clean cut than the virulent. We, therefore, postponed the consideration of the former until we had established, to our satisfaction, the relationships of the latter.

From a practical point of view the main value of this method of investigation should be found in epidemiology. In agreement with Durand and Guérin, our work suggests that, broadly speaking, in one epidemic one type prevails. This fact is also eloquently shown by the results of Langer, Mason and others.

SIMULTANEOUS AND REPEATED SWABS.

Positive cultures from the nose and throat of the same patient at the same time have in our hands always shown the same type of organism. Organisms cultured from the same patient at different times have almost always shown the same type of organism. We have followed several individuals in this way for many months, and this serological constancy is the rule in the vast majority of cases. We have, however, met with a few interesting variations. Thus one patient with a persistent otorrhea gave us one strain allied to types III and IV and a month later another mongrel 502 (*vide* Tables III and IV). Likewise, of six swabs from one patient, three were pure type III and three mongrel III and IV. This variability has been specially marked with type III carriers. Thus we find III and IV, III and V.

We have also found type II followed by VII and V; in one case we found IV followed one month later by V.

As we pointed out before, we are dealing mainly with an area in which type III is dominant; we should therefore expect the mongrel strains to show their relationship to this type.

FAMILIES.

Organisms cultivated from members of the same family, and contacts of one case show in general the same type organism. We have examples of this from types II, III and V. No variation between types has been found in one family at one time; the variation may occur later in the organisms cultivated from individual members.

EPIDEMICS.

We have examined series of organisms from two sources. The first is a school near London in which an epidemic was followed by sporadic cases. The second is a London borough, the organisms being isolated mainly from direct contacts, but including a few cases. The results are best arranged chronologically as under:

The Institution gives 23 virulent cultures of which

15 are type III; 1 is type IV; 4 are type V; 3 are type IX:
all the avirulent cultures examined were nasal and type V.

The London Borough series gives 33 organisms all virulent, all from the throat:

29 are type III; 1 a mongrel III and IV; 3 are type V.

Our material so far is scanty, but it suggests that, though an epidemic may start with one type, other types will appear. This also would be expected if we believe that the agglutinative types of *B. diphtheriae* are not fixed, but to borrow Prof. Andrewes' expression, "kaleidoscopic."

The typing of the few avirulent cultures in the School series is also of interest as suggesting their nearness of kin to the prevailing virulent type. Most of these nasal cases were regarded by us as carriers.

SUMMARY.

1. Evidence is given that there exists a multiplicity of serological groups of *B. diphtheriae*.

2. Ten of these groups have been studied in this communication.

3. Into these ten groups could be placed all except 16 of 348 strains of virulent *B. diphtheriae* investigated.

4. A description of the technical difficulties encountered is included in the hope that this may prove of service to other workers.

Some evidence on the antigenic structures of different strains is advanced.

5. The application of this method of investigation to the problems of epidemiology is briefly discussed, and evidence of its value brought forward.

Table I. *Direct agglutination.*

	Types										3 and 4	Un- classified	Total
	1	2	3	4	5	6	7	8	9	10			
Cases	4	5	32	4	20	2	2	2	6	1	3	3	84
Persistents	1	0	4	1	5	0	0	0	0	0	1	0	12
Contacts	0	0	26	1	3	0	0	0	0	0	1	0	31
Carriers	6	20	80	6	31	8	5	4	1	8	7	13	189
Unknown	11	4	3	2	2	0	0	0	0	0	1	9	32
Total	22	29	145	14	61	10	7	6	7	9	13	25	348
310											13	25	
323												25	

Table II. *Final results.*

											3 and 4	Un- classified	Total
	1	2	3	4	5	6	7	8	9	10			
Cases	4	5	33	4	21	2	2	2	6	1	2	2	84
Persistents	1	0	4	1	5	0	0	0	0	0	1	0	12
Contacts	0	0	26	1	3	0	0	0	0	0	1	0	31
Carriers	6	20	83	7	31	8	5	5	1	8	5	10	189
Unknown	14	5	3	2	2	0	0	1	0	0	1	4	32
Total	25	30	149	15	62	10	7	8	7	9	10	16	348
332													

Table III. *Direct agglutination.*

Sera		Antigens										519					
		1	2	3	4	5	6	7	8	9	10	301	502	525	506	46	666 668 77
1	34, Δ79, Δ85	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
2	26	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
3	55, 325, 477	-	-	+	-	-	-	-	-	-	-	1/2	-	1/5	+	-	-
4	Δ 90, 663	-	-	-	+	-	-	-	-	-	-	1/40	-	1/40	-	-	-
5	36, 186, 317, Δ 91, 296	-	-	-	-	+	-	-	-	-	-	-	-	1/16	-	-	-
6	459, 393	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
7	516, 227, 784	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
8	61, 98, 613	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
9	851	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
10	35	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
"Mongrel"	301	1/16	-	+	1/16	1/16	-	1/16	-	-	-	+	-	1/8	+	-	-
	502	+	-	-	-	-	-	1/4	1/16	-	-	-	+	-	-	-	-
	525	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	506	-	-	+	1/2	-	-	1/8	-	-	-	+	-	1/32	+	-	-
Unknown	519, 46	-	+	-	-	-	-	1/8	-	-	-	-	-	-	-	+	-
	666	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	668	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

- = No agglutination at 1/20 final dilution

+ = Full.

1/2, 1/4, etc. = Fraction of full titre.

Table IV. *Absorptions*

	1	2	3	4	5	6	7	8	9	10	301	502	525	506	519 46	666	668	77
1	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
2	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
3	-	-	+	- or 1/4	-	-	-	-	-	-	1/2	-	-	-	-	-	-	-
4	-	-	- or 1/2	+	-	-	-	-	-	-	-	-	+	1/2	-	-	-	-
5	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
301	- or 1/2	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
502	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
525	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
506	-	-	+	-	-	-	-	-	-	-	(+)	-	-	+	-	-	-	-
519, 46	-	+	-	-	-	-	(+)	-	-	-	-	-	-	-	+	-	-	-
666	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
668	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = full absorption.
(+) = almost complete absorption.
1/2 and 1/4 denote relative completeness, compared with homologous strain.

Table V. *Table of comparative results.*

1	2	3	4	5	6	7	8	9	10		
+	-	-	-	-	-	-	-	-	-	Nicholas and C.	A
+	-	-	-	-	-	-	-	-	-	Havens: type I	B
-	+	-	-	-	-	-	-	-	-	" " II	B
+	tr.	tr.	tr.	1/16	1/16	1/8	1/8	?	?	Bell " I	C
1/8	+	-	-	-	-	-	1/8	?	?	" " II	D
-	-	tr.	-	+	1/16	1/4	-	?	?	" " III "Blackburn"	D
-	-	+	-	tr.	1/4	+	-	?	?	" " III "Andrews"	D
-	-	+	-	-	-	-	-	?	?	" " III "Mulaney"	D
+	-	-	-	-	-	-	-	-	-	Durand (from Park): type I	C
-	-	-	-	-	-	-	-	-	-	" " " I A.V.	B
-	-	-	+	-	-	-	-	-	-	" " " III	B
-	-	-	+	-	-	-	-	-	-	" " " IV	C
-	-	-	-	-	-	-	-	-	-	" " " V	B

A = deduced from literature.
B = culture tested.
C = serum and culture tested.
D = serum tested.
A.V. = avirulent.

Table VI. *Horse sera (agglutinations).*

	Type cultures										
	1	2	3	4	5	6	7	8	9	10	
H 1154. Type I	-	-	-	-	-	-	-	-	-	-	23. vii. 22 before inj.
	1000	tr.	-	-	-	-	-	-	-	-	28. viii. 22
	800	-	-	-	-	-	-	-	-	-	6. ix. 22
	2000	-	40	10	-	-	-	10	50	-	11. ix. 22
	2000	150	80	tr.	80	-	tr.	-	80	20	22. ix. 22
	640	30	10	-	-	-	-	-	20	-	3. x. 22
H 1156. Type II	-	-	-	-	-	-	-	-	-	-	28. vii. 22 before inj.
	64	500	128	-	25	-	-	-	48	-	28. viii. 22
	-	1000	60	-	tr.	-	-	30	60	-	6. ix. 22
	tr.	40	40	20	10	tr.	15	15	20	-	11. ix. 22
	30	2000	100	30	40	20	20	40	40	-	22. ix. 22
	-	2000	100	-	30	-	-	-	40	-	3. x. 22
H 1173. Type III	-	-	-	-	-	-	-	-	-	-	28. vii. 22 before inj.
	-	-	1500	-	-	-	-	-	-	-	28. viii. 22
	-	-	1500	-	-	-	-	-	-	-	6. ix. 22
	-	50	800	tr.	tr.	-	tr.	20	-	-	11. ix. 22
	20	60	1000	10	40	tr.	-	30	30	tr.	22. ix. 22
	-	-	1000	-	-	-	-	-	-	-	3. x. 22

Table VII. *A school.*

Date	Types of organisms occurring in a prolonged series of cases					
	Virulent	A.V.	3	4	5	9
14. ii. 22	2	0	2	0	0	0
15. ii. 22	2	0	2	0	0	0
19. ii. 22	1	0	0	0	1	0
21. ii. 22	1	0	1	0	0	0
24. ii. 22	2	0	2	0	0	0
27. ii. 22	1	0	0	0	0	1
6. iii. 22	1	0	0	0	0	1
16. iii. 22	1	0	0	1	0	0
27. iii. 22	1	0	0	0	1	0
10. iv. 22	1	0	1	0	0	0
10. iv. 22	0	2	0	0	2	0
11. iv. 22	0	1	0	0	1	0
13. iv. 22	0	1	0	0	1	0
18. iv. 22	2	0	1	0	1	0
25. iv. 22	1	0	1	0	0	0
27. iv. 22	1	0	1	0	0	0
1. v. 22—2. vi. 22	4	0	4	0	0	0
23. vi. 22	1	0	0	0	1	0
24. viii. 22	1	0	0	0	0	1
Total	23	4	15	1	8	3

Table VIII. *A London Borough.*

Date	Cases	Contacts	Type		
			3	3 and 4	5
September and October, 1921	4	2	6	0	0
November, 1921	0	16	12	1	3
December, 1921	0	2	2	0	0
January, 1922	0	3	3	0	0
February and May, 1922	0	2	2	0	0
July, 1922	0	4	4	0	0
Total	4	29	29	1	3

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THE ATMOSPHERE OF THE UNDERGROUND ELECTRIC RAILWAYS OF LONDON.

A STUDY OF ITS BACTERIAL CONTENT IN 1920.

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(With 1 Text-figure and 6 Charts.)

CONTENTS.

	PAGE
I. Introduction	123
II. Method of procedure in collection and examination of samples of railway air	124
III. Summaries of bacteriological counts obtained on the six underground railways and in the open air	127
IV. General consideration of bacteriological results and comparison with observations of previous workers	130
V. Relationship of the number of organisms in railway carriage air to passenger density	136
VI. An "Index of bacterial pollution" of railway carriage air	141
VII. Species of organisms found in the air of underground railways, with reference in particular to the group of moulds	142
Summary and Conclusions	150
References	152
Comparative Block Charts III—VI	153

I. INTRODUCTION.

FOR the purpose of ascertaining, so far as possible, the bacterial content prevailing in the air of the London Electric Tube railways, investigations were undertaken during the first half of 1920. The general object aimed at was to obtain information as to the degree of bacterial air contamination to which passengers were exposed in the course of daily travel and to learn how far the means provided for ventilation of the carriages and underground railways were effective from this point of view.

With the recent severe influenzal epidemics of 1918 and 1919 still fresh in the mind, there was also coupled the view that overcrowding and insufficient carriage ventilation were contributory factors in the spread of infection in the past and likely to be so again in the event of a recurrent epidemic, which then threatened but did not materialise.

Among previous publications on atmospheric pollution available for reference and guidance, particular mention must be made of the work of F. W. Andrewes on the Atmosphere of the Central London Railway in 1902, contained in his Bacteriological Report to the Parliamentary Committee of

the London County Council⁽¹⁾. So appropriate to the work in hand, this investigation proved invaluable, as will appear evident from frequent comparison and quotations made in subsequent pages of the present report. Use has also been made of the Reports by Carnelly, Haldane and Anderson on the Air of Dwellings and Schools (1887)⁽²⁾ and by Graham-Smith on the Micro-organisms in the Air of the House of Commons in 1902, reprinted in the *Journal of Hygiene*, 1903⁽³⁾. At a later date (1906) was published Mervyn Gordon's Bacteriological Report⁽⁴⁾ in Section IV of the *Investigation of the Ventilation of the Debating Chamber of the House of Commons* (Cd. 3068) to which further reference will be made. More recently, in 1908, there appeared in book form a comprehensive study of the *Air and Ventilation of Subways*, by G. A. Soper, Ph.D.⁽⁵⁾, which includes a review of the air conditions of the various European subways used for railway passenger traffic and gives an account of the particular investigations carried on in the New York subways in 1904—the basis of the publication.

The comparisons of the conditions reported, notably in the Central London Railway (Andrewes, 1902), the Metropolitan Railway of Paris (1901) and the New York Subways (1904), do not, in Dr Soper's opinion^(5 a), afford criteria for fixing a definite standard of purity for the air of subways, which, he says, "should be kept as pure as necessary to meet the sanitary requirements of the particular place in question—in other words each subway should be considered on its own merits." He observes also that, such subways as the Metropolitan of London and Rapid Transit of New York more nearly resembling streets than buildings, the standards regarded as suitable for the latter are not sufficiently exacting for superficial subways, whereas for the deep tubes of London, far below the streets, it is desirable to raise a high standard of purity.

II. PROCEDURE ADOPTED FOR EXAMINATION OF AIR OF LONDON TUBE RAILWAYS.

The following six Electric Railways were made the subject of investigation:

1. Central London.
2. City and South London.
3. Bakerloo.
4. Piccadilly and Brompton.
5. Hampstead and Highgate.
6. The Inner Circle of the Metropolitan District.

It was considered advisable that efforts should be directed to obtaining samples of air at times when the carriages and platforms were most likely to be crowded, *i.e.* during the rush hours and preferably between 5 and 7 p.m. In order that atmospheric conditions governing the time of year might not prejudice the results, journeys were made alternately on the various railways, rather than consecutively on each railway in turn. On the days corresponding with the tests carried out on the railways, samples for purposes of comparison

were also taken of the open air at points more or less in the neighbourhood of the railway concerned, but not invariably so.

Certain preparatory and trial samples for bacteriological examination were taken during the month of February but the investigations proper, the results of which are contained in this report, were not begun until March 3rd and were concluded on May 31st, 1920.

Through the courtesy of the authorities of the London Electric Railway Company, by the grant of special free passes and letters of introduction, every facility was afforded to the work of collecting the samples of air on the trains and platforms.

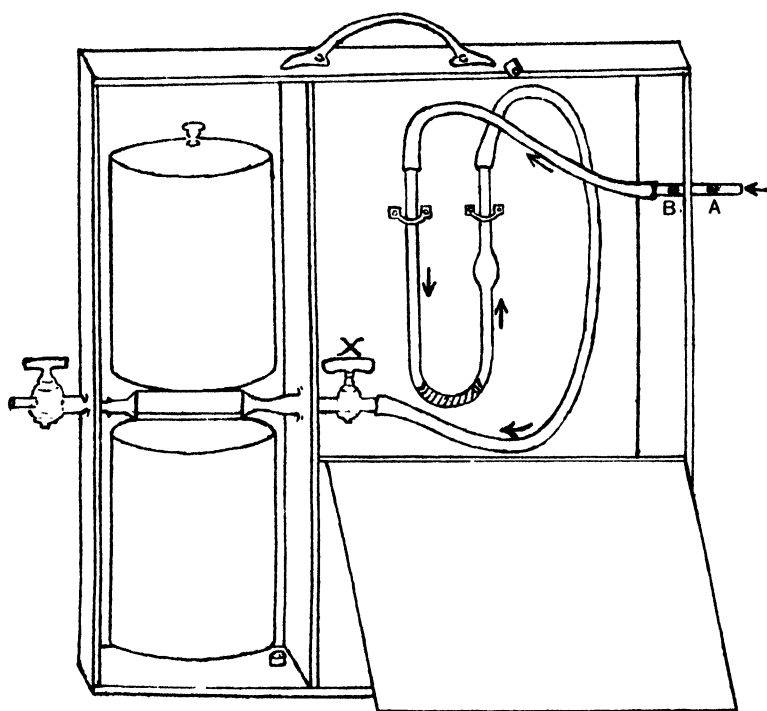
The majority of samples were taken while the trains were in transit and during the most crowded hours of the evening, *i.e.* between 5 and 7 p.m. A total of 60 samples of the air in the carriages was collected, *i.e.* ten from each railway, and alternate samples were examined by plate culture for organisms growing at 37° C. (mesophil group) and for those growing at 20° C. (psychrophil group). Therefore from each railway in all five samples were examined at 37° C. and five samples at 20° C. A total of 22 samples was also collected on the platforms of the most crowded underground stations of the various railways, excepting the District Railway, during the rush hour. Forty samples were taken of the outside air in the following localities: Charing Cross Gardens, Temple Gardens, steps of 2 Savoy Hill, the roof of Savoy Hill Buildings, the top of Duke of York's steps, Pall Mall, in Hyde Park, near Marble Arch Entrance, near the Serpentine and near Hyde Park Corner Entrance, also on Hampstead Heath and on Clapham Common.

			No. of samples
Railway carriages	60
Station platforms	22
Station passages	2
Outside air	40
Total			124

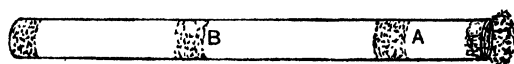
Of this total, nine samples, seven from carriage air and two from platform air, were rejected owing to unreliable cultural results due to confluence of colonies or liquefaction of culture media; thus leaving a net total of 115 samples (75 of Underground Railway Air and 40 of Open Air) on which the whole investigation is based.

Method of Collection of Samples of Air for Bacteriological Analysis. For the purpose of collecting the organisms contained in a known volume of air, an aspirator constructed on the hydrostatic principle was made use of attached to a special glass sampling tube. The method adopted was in principle that introduced by Frankland⁽⁶⁾, subsequently used by Haldane and Laws and more recently by Andrewes and Graham-Smith, namely, the aspiration of a known volume of air through a plug of glass wool which retains all micro-organisms and can subsequently be distributed through a suitable cultivating medium in a glass capsule. The volume of air which had been

found most convenient and suitable for investigation was 5 litres, a larger quantity being liable to yield a number of organisms too great for accurate counting. The sampling tube (*AB*) consisted of a glass tube, 5 inches in length and one-third inch in diameter, containing two plugs of pounded glass wool placed at about one-and-a-half inches from either end of the tube, the one (*A*) firmly compressed to a quarter of an inch in thickness, the other (*B*) less compact. The ends of the tube were also plugged with cotton wool to prevent



Hydrostatic Aspirator



Sampling Tube (Half Actual Size)

Fig. 1.

the introduction of accidental contaminations in handling and connecting up with the rubber tubing. Before use the plugged sampling tube was carefully sterilised by heating in the dry oven to 160°C . for an hour, and kept in a plugged sterile test tube until required.

The aspirator employed was of familiar pattern, consisting of two connected brass cylinders each of 1 litre capacity, one being filled with water, and the two rotating on a hollow axis, which communicated by means of a stop-cock (*X*) and rubber tube with a glass U tube; the latter was connected

by rubber tubing to the glass sampling tube containing its two plugs of powdered glass wool—*A* and *B*. The whole was enclosed in a wood framework measuring $15 \times 16 \times 4\frac{3}{4}$ ins., weight 15 lbs., and being quite portable was convenient for use in the railway carriages. Aspiration was effected by rotating the double cylinder, thus allowing the flow of water from the upper to the lower half and with it the suction of air through the plugs in the sampling tube to the emptying cylinder.

Count was kept of each rotation corresponding with the aspiration of 1 litre of air, and the sampling tube remained in place till the required 5 litres had been aspirated. The tube was then carefully detached, the ends replugged with sterile wool, and transferred to the sterile test tube for return to the laboratory.

The cultivation of organisms caught in the glass wool plugs from the air aspirated through the sampling tubes was effected in the following manner:

Each of the two plugs was projected into separate sterile glass capsules by means of a sterile glass rod driven down the lumen of the tube. The plug was then thoroughly broken up with sterile wire loops under cover of the capsule-lid. Melted agar-agar or gelatin, according to whether cultivation at 37° C. or 20° C. was to be applied, was poured quickly over the lower half of the capsule, and the teased glass wool and melted culture medium were thoroughly mixed by inclining the capsule slightly from side to side. When the medium had completely set, the agar plates were incubated at 37° C. and the gelatin at 20° C. Incubation was allowed to go on for six days, careful inspection being made each day to watch for development of colonies, which were "ringed" as they became visible, and finally counted. The aggregate of colonies thus obtained in each total count of two capsules represented the number of organisms derived from 5 litres of air. As was to be expected it was found that the capsule inoculated with the plug nearest the free end of the sampling tube yielded by far the greater bulk of colonies, it being exceptional to find more than a very few colonies in the capsule containing the second or inner plug. The counts of colonies having been tabulated, subcultures were then made from a certain proportion. With the application of further cultural tests and by the morphological and staining characters of the resulting growths, as described under Section VII, an attempt was made to classify the various organisms isolated.

III. SUMMARIES OF THE RESULTS OBTAINED ON THE SIX UNDERGROUND RAILWAYS.

1. THE CENTRAL LONDON RAILWAY.

The atmosphere of the Central London Railway having been bacteriologically examined in 1902 by F. W. Andrewes, the results obtained in 1920 were of particular interest after the lapse of 18 years during which the conditions above ground had changed considerably owing chiefly to the replacement of horse traffic by motor traffic.

128 *Atmosphere of Underground Electric Railways*

Summary of bacteriological counts of colonies from 5 litre samples of air from the Central London Railway.

				No. of colonies growing in agar-agar at body temperature	Colonies growing in gelatin at room temperature
Railway carriages	{ 7	—
				{ 9	—
				{ 17	37
				{ 16	—
				{ 13	17
				—	57
Average	12.4	37
Platforms	{ 28	40
				{ 13	40
Average	20.5	40
Total averages of carriage and platform air ...				14.7 at 37° C.	38.2 at 20° C.
Averages of open air controls on corresponding dates ...				11.7	20.6 per 5 litres

These results for the Central London Railway may be compared with those obtained by Andrewes in his examination of 12 samples of the air in the railway carriages, platforms, passages, lifts and tunnels of the same railway during March to June, 1902.

Total Averages (1902).

- (1) Colonies at 37° C. per 5 litres of air 6.9.
- (2) Colonies at 20° C. per 5 litres of air 44.1.

It should be noted that crowded conditions were selected for the observations of the present survey, and that the culture media were given a period of six days, which will partly account for the higher counts obtained of colonies developing at body temperature, whereas in the conditions during Andrewes' observations there was no excessive crowding of passengers, and his plates were not incubated for longer than four days.

2. CITY AND SOUTH LONDON RAILWAY.

Summary of bacteriological counts of colonies from 5 litre samples of air from the City and South London Railway.

				No. of colonies growing in agar-agar at body temperature	Colonies growing in gelatin at room temperature
Railway carriages		{ 24	—
				{ 5	—
				{ 21	49
				{ 32	71
				{ 6	29
Average	17.6	49.6 per 5 litres
Platforms	{ 10	—
				{ 8	47
				{ 21	37
Average	13	42 per 5 litres
Total averages of carriage and platform air ...				16	46.6
Averages of open air controls on corresponding dates ...				8.5	36

3. BAKERLOO RAILWAY.

Summary of bacteriological counts of colonies from 5 litre samples of air on the Bakerloo Railway.

	No. of colonies developing on agar-agar plates at body temperature			No. of colonies developing on gelatin at room temperature
Railway carriages	{ 14 7 11 40 13			— 48 36 52 24
Average	17			40
Platforms	{ 17 26			71 36
Average	21.5			53.5
Total averages of carriage and platform air	18.3			44.5
Averages of open air controls on corresponding dates ...	8.2			36

4. PICCADILLY AND BROMPTON RAILWAY.

Summary of bacteriological counts of air samples on the Piccadilly and Brompton Railway.

	No. of colonies developing at body temperature 37° C.			No. of colonies developing at room temperature 20° C.
Railway carriages	{ 31 18 18 23 20			42 52 30 40 —
Average	22			41
Platforms	{ 21 37			56 —
Average	29			—
Total averages of carriage and platform air	24			44
Averages of open air controls on corresponding dates ...	13.6			41.6

5. HAMPSTEAD AND HIGHGATE RAILWAY.

Summary of bacteriological counts of colonies obtained from the air of the Hampstead and Highgate Railway.

	No. of colonies developing at body temperature 37° C.			No. of colonies developing at room temperature 20° C.
Railway carriages	{ 47 11 20 18 29			33 49 45 37 —
Average	25			41
Platforms	{ 35 15			60 23
Average	25			41.5
Total averages of carriage and platform air	25			41.2
Averages of open air controls on corresponding dates ...	8			32

6. METROPOLITAN DISTRICT. (INNER CIRCLE.)

Summary of bacteriological counts of colonies obtained from 5 litre samples of air on the Inner Circle of the Metropolitan District Railway.

				No. of colonies developing at body temperature 37° C.	No. of colonies developing at room temperature 20° C.
Railway carriages		10	32
				10	—
				13	—
				22	30
				53	70
				40	53
Average	24.6	46.2
Averages of open air controls on corresponding dates				16	23.5

IV. GENERAL CONSIDERATION OF THE BACTERIOLOGICAL RESULTS.

From the foregoing summaries of the different observations made on the various railways it may be noted that taking the total averages of the bacteriological counts the following results are obtained:

No. of samples: 53			(1) For railway carriages.					
at 37° C.		at 20° C.	No. of organisms growing at 37° C. (mesophil)*			No. of organisms growing at 20° C. (psychrophil)*		
			Averages†	Highest	Lowest	Averages†	Highest	Lowest
5	3	Central London	12.4	17	7	37	57	17
5	4	Bakerloo	17	40	7	40	52	24
5	3	City and South London	17.6	32	5	49.6	71	29
5	4	Piccadilly and Brompton	22	31	18	41	52	30
5	4	Hampstead and Highgate	25	47	11	41	49	30
6	4	Metropolitan District (Inner Circle)	24.6	53	10	46.2	70	30
No. of samples: 20			(2) For platforms.					
3	2	City and South London	13	21	8	42	47	37
2	2	Central London	20.5	28	13	40	40	40
2	2	Hampstead	25	35	15	41.5	60	23
2	2	Bakerloo	21.5	26	17	53.5	71	36
2	1	Piccadilly and Brompton	29	37	21	56	56	56
Metropolitan District			No tests made			No tests made		
(3) Total averages for railway carriages and platforms.								
Central London			14.7	38.2				
City and South London			16	46.6				
Hampstead and Highgate			25	41.2				
Bakerloo			18.3	44.5				
• Piccadilly and Brompton			24	44				
Total average			23.2	45.3				

* The differentiation of the mesophil (37° C.) and psychrophil (20° C.) organisms into separate groups does not constitute a hard and fast distinction between the two, for the mesophil group, enumerated as such, may include a proportion also of the psychrophil organisms. But the majority of pathogenic organisms proper will be strictly mesophil, only growing at body temperature.
† per 5 litres of air.

The result of the samples taken at the foot of the escalator at Oxford Circus Station serving both the Central London and Bakerloo Railways is not included in the above total average (45.3). This observation yielded the highest

count obtained anywhere, viz. 51 colonies at body temperature (37° C.) and 110 colonies at room temperature (20° C.). It was taken under conditions of passenger density and air movement likely to yield a high rate of bacterial contamination.

Open air controls. Observations made on dates corresponding with those on the various railways yielded the following averages:

Dates corresponding with observations on the	Railways	Organisms at 37° C. (per 5 litres)	Organisms at 20° C. (per 5 litres)
	Central London	11·7	20·6
	Bakerloo	8·2	36
	City and South London	8·5	36
	Piccadilly and Brompton	13·6	41·6
	Hampstead and Highgate	8·0	32
	Metropolitan District	16·0	23·5
Averages of all open air controls		11·0	31·6
At 37° C.	Highest 31 (Hyde Park)		
	Lowest 2 (Hampstead Heath)		
At 20° C.	Highest 85 (Hampstead Heath)		
	Lowest 6 (Roof of Savoy Hill Buildings)		

The combined averages of results obtained from all railways yielded:

	Organisms at 37° C. (per 5 litres)	Organisms at 20° C. (per 5 litres)
For carriage air	19·8	42·8
For platform air (including the result of sample taken at Oxford Circus escalator between the Bakerloo and Central London Stations) ...	26·6	52
For carriage and platform air	23·2	45·3

As a large proportion of the organisms present in the air do not grow at the body temperature (37° C.), the estimate of total number must be drawn from a consideration of the counts obtained at room temperature (20° C.).

It will be seen from the figures given above that the Central London Railway yields the lowest total average of organisms for railway carriage air (37 per 5 litres); the Bakerloo comes next with 40, followed by the Hampstead and Piccadilly Railways with 41 each, and then the Metropolitan District with 46·2, and lastly the City and South London with 49·6. In the platform samples, too few perhaps to provide reliable data, the counts varied from an average of 40·0 for the Central London to 56 per 5 litres of air for the Piccadilly Railway. The higher range of the bacterial content is to be attributed to the greater draught and dust disturbance prevailing on the platforms.

Grouping together both carriage and platform results the total averages place the Central London first with 38·2, then the Hampstead Railway with 41·2, the Piccadilly follows next with 44, and the Bakerloo with 44·5; lastly the City and South London Railway with 46·6. Owing to the Metropolitan District Stations all being directly open to the outside air no platform samples were taken on the Inner Circle.

The average counts of total organisms in the open air obtained on corresponding dates from open air controls are found to vary from 20·6 to 41·6

per 5 litres, *i.e.* a degree of microbic air pollution not greatly behind that found in railway air and in one group actually slightly above the corresponding railway averages; the total means appear as 31.6 for open air and 45.3 for railway air.

The comparison is, however, less close when the figures of average results for organisms growing at body temperature are considered. That the disparity between railway and open air counts should be more marked in the body temperature or mesophil group is not surprising, in view of the usually crowded conditions under which railway samples were taken, favouring a greater proportion of organisms growing at the higher temperature. Taking the average of all results the differences between open air and railway air appear more evident but are still not as great as might perhaps have been expected.

Open air and railway air compared.

	Open air	Railway air	
No. of organisms obtained at body temperature	11	23.2	} per 5 litres
Ratio	1	2	
No. of organisms obtained at room temperature	31.6	45.3	} per 5 litres
Ratio	10	14.5	

Comparison of the results of the recent observations with those of other workers. Comparison may here be drawn between the figures above quoted and the results obtained by other observers, notably Andrewes in 1902. Between the figures derived from the latter's report and those of this paper there is found to be extraordinarily close agreement for the total averages of the number of organisms growing at room temperature particularly in the case of railway air. For the number of organisms growing at body temperature in the two series of observations the *ratio* between open air and railway air is also very much the same, *viz.* as 1 to 2, but in the case of the recent observations the actual total at body temperature is some three or four times higher for both open and railway air. This increase, at any rate for railway air, may, as already suggested, be accounted for in part by the crowded conditions existing at the time the samples were taken. Such a hypothesis can scarcely be put forward, however, to explain the increase in the number of organisms growing at body temperature in the open air. It would be a daring suggestion to throw out that the increase in the latter may be due to the vast development in human and motor traffic, compared with what existed 18 years ago, and consequently greater microbic air pollution, despite a diminution in horse traffic.

The total averages of the respective observations are as follows:

	Open air		Average of six Tube Railways 1920 (J.G.F.)	Central London Railway air 1902 (F.W.A.)
	1902 (F.W.A.)	1920 (J.G.F.)		
No. of organisms growing at body temperature (37° C.)	3.4	11.0	23.2	6.9 per 5 litres
No. of organisms growing at room temperature (20° C.)	33.9	31.6	45.3	44.1 „

If the results of the two series of observations obtained in the Central London Railway are alone compared there is, however, not quite so close

an agreement between the number of organisms growing at the two temperatures.

Average of carriage and platform samples on the Central London Railway obtained in the recent observations	
Organisms growing at body temperature	14.7 per 5 litres
Organisms growing at room temperature	38.2 „

A comparison may also be made with the results of Graham-Smith's(3) experiments on the air of the House of Commons during July, 1902.

Place	No. of organisms per litre at 20° C. varying between
In chamber during debate on July 21st	10.6 and 4.4
Ventilating shaft to chamber	2.6 and 0.8
Committee, Dining and Smoking Rooms of the House of Commons	44.2 and 20.9
Outside air, July 18th	6.0 and 1.5 (according to altitude)

The air of the open space surrounding the Houses of Parliament contained 4.2 organisms per litre which he regarded as a comparatively small number. The mean of his experiments, numbering 11 in the Debating Chamber, was 5.8 per litre. This, from the bacteriological point of view, he described as remarkably pure; the mean of six experiments in the Committee, Dining and Smoking Rooms was considerably higher (32.3 per litre).

The mean of Andrewes' results at 20° C. reduced to the same proportion per litre yielded for

(1) Central London Railway air	...	8.82 per litre
(2) Outside air	6.78 „

The mean of the 1920 observations reduced to the same proportion per litre yielded for

(1) Railway air of all the six railways	...	9.00 per litre
(2) Outside air	6.32 „

The results of these later observations, when viewed in comparison with the findings of Andrewes and Graham-Smith, may be regarded on the whole as satisfactory evidence of no really gross microbic air pollution in the various railways concerned.

The results of observations at an earlier date may be quoted from the work of Carnelly, Haldane and Anderson (7) in Dundee dwelling-houses and schools, published in the *Philosophical Transactions*, 1887, and by Carnelly (1893-4) (8). They reported on the influence of locality, age of the buildings, cleanliness and ventilation. In 28 naturally ventilated schools described as very dirty a mean of 152.1 organisms per litre was obtained, but in 18 schools in which mechanical ventilation was in use the average number of organisms amounted only to 16.58 per litre. Where, however, better conditions of cleanliness existed such as in private schools, though naturally ventilated, the degree of microbic pollution was found to be very considerably lower, viz. nine organisms per litre. A still lower figure prevailed in good class and mechanically ventilated institutions where cubic space was much greater, such as 2.8 and 3.6 per litre in the Dundee University College and High School respectively.

The influence of cubic space, general cleanliness and number of occupants in the bacterial air content was also shown by the results of observations made in various dwelling-houses.

The air of one-roomed houses yielded an average of 60 organisms per litre, of two-roomed houses 46, and of four or more roomed houses nine only per litre. The results of their observations of outside air varied between 0·8 per litre for quiet places and 17·5 per litre in busy streets. A standard of air purity was suggested for dwelling-houses and schools and it was considered that a limit of 20 organisms per litre should not be exceeded.

In the samples taken of railway air it will be noted that the results are well within this limit and only once exceeded it, in the case of the air examined at the foot of the Oxford Circus escalator, when 22 organisms per litre were obtained.

It should be pointed out, however, that the standard of purity advocated of 20 organisms per litre is based on results derived from observations taken under conditions of atmospheric pollution which were unhesitatingly condemned. Moreover, this standard a quarter of a century ago was probably regarded as presenting an improvement within the then bounds of possible attainment.

There are grounds for the belief that such a limit at the present day cannot be considered satisfactory or sufficient.

The observers qualified their standard as applicable only to the method then employed in the use of Hesse's Tube (measuring 70 cm. by 3·5 cm.) coated with Koch's jelly, over which air was aspirated at the rate of 1 litre in three minutes. Their readings of the number of resulting colonies also extended over a period of three to four weeks at room temperature for each examination made. An improved and somewhat more accurate and practicable method was introduced by Frankland⁽⁶⁾ who conducted a number of experiments on the air outside the Science Schools, South Kensington. He aspirated measured quantities of air through a narrow glass tube containing sterile glass-wool plugs, such as was used by Andrewes in 1902, and has been adopted in the present investigations as likely to give the most reliable results. Frankland's control experiments with both the Hesse Tube and the narrow tube plugged with glass wool showed on the whole remarkable agreement between the results of the two methods, when conducted in the still atmosphere prevailing in a closed room; howbeit, the Hesse method was regarded as yielding results generally slightly too high—on the other hand, under conditions of disturbed air and free currents in active movement such as obtained outside, Frankland estimated the Hesse Tube to yield considerably too high a figure, which required correction by deduction of one-third of the number of organisms per litre—19 of Frankland's original experiments on the outside air yielded an average of seven organisms per litre. With further tests by a later method applied to some 40 observations he obtained an average of between 4 and 2·5 per litre for the air outside the Science School Laboratories and in examinations

made outside St Paul's Cathedral his results averaged 1 per litre at the top of the Dome, 3.8 per litre at the base of the Dome and 4.7 per litre in St Paul's Churchyard.

The average of Graham-Smith's figures for the air outside the Houses of Parliament varied between 1.5 and 6 per litre, according to altitude.

Andrewes' average for London air in 1902 was 6.78, and the mean of the recent open air tests in various parts of the Metropolis was 6.32 per litre.

In the bacteriological investigations of the air of New York subways in 1903, about 3000 samples were examined. They included numerous exposures of plate culture media for periods of 15 minutes or less, and estimations derived from the filtration of measured quantities of air through sand filters, taken in the subways (but not in the cars), and in the open air of the streets outside. They yielded numerical results the exact opposite of those found both in 1902 and 1920 in the London investigations, *i.e.* an average ratio of two organisms in the street air to one in the subway air; plate exposure providing an average of 1150 organisms, growing at body temperature, in street samples and 500 in subway air; and the filtration method giving 6500 organisms per cubic metre in the streets and 3200 per cubic metre in the subways^(5b). The very different conditions, atmospheric and constructional, pertaining to the London and New York tube systems and streets, do not, perhaps, afford a fair basis for comparison of the respective ratios of the bacterial contents of the street and tunnel atmospheres of the two Capitals, and, as already quoted, "each subway should be considered on its own merits." The New York report includes studies of the possible action on bacteria of lubricating oils (in use on the running machinery) and of the deodorants and disinfectants, employed on platforms and in conveniences, but found to be of negative bactericidal value. Also, many analyses of dusts and numerical determinations of the bacteria, present in the dust of subways, were made and compared with dust samples from theatres, hotels and elsewhere. Detailed examinations of this character were, however, quite outside the range of the present investigation.

Dr Soper in his conclusions^(5c) was of opinion that according to usual standards based on chemical and bacteriological analyses the general air of the New York subways (not of the cars) was always and everywhere satisfactory; the exchange of air was abundant except in closed and crowded carriages and where dense crowding on platforms occurred.

The high temperature of the subways was the most evidently objectionable feature, especially in the morning and evening rush hours, the heated air not escaping with sufficient rapidity for comfort, although renewed often enough for health.

He regarded the bacteriological condition of subway air as satisfactory in that only half as many bacteria were found as in the air of the streets, although too much reliance was not to be placed upon this comparison as a guide to the condition of the subway air.

V. RELATIONSHIP OF THE NUMBER OF ORGANISMS IN THE AIR
OF RAILWAY CARRIAGES TO PASSENGER DENSITY.

At the time of collection of air samples in the carriages approximate estimations of the number of travelling passengers were made and grouped under the head of passenger density (*vide* Charts I and II) as "packed," "half standing," "standing," "full," " $\frac{3}{4}$ full," " $\frac{1}{2}$ full," " $\frac{1}{4}$ full," and "empty," numerically represented for purpose of comparative charting by the figures 100, 71, 64, 54, 40, 27, 14 and zero, 54 being the average of the total seat accommodation in a single compartment.

The observations were made with a view to tracing relationship between passenger density, particularly overcrowding, and the bacterial content of the air prevailing at the time in the moving trains. In discussing any such possible correlation it is necessary to bear in mind that a high bacterial content is associated with dust disturbance due to fluctuating air currents, in the absence of which floating microbic particles will tend to subside with a gradual lowering of the bacterial air content. The process of settling, however, is extremely slow since the weight of a bacterium is less than a billionth of a gram and it may be held in suspension for considerable periods (9).

In the air of a compartment which has been emptied, provided the air remains still, the degree of microbic pollution will steadily diminish—the more quiescent the state of air the greater will be the subsidence of dust and the reduction in the number of micro-organisms in suspension in the air.

A condition of absolute stillness of the air can, however, never be found in a moving train and would only be possible when stationary and with windows and ventilators closed.

By charting the mean of all observations made consecutively and under corresponding conditions of passenger density for the respective groups of mesophil and psychrophil organisms, an attempt has been made (Chart I) to

Tabulated results of the total averages showing comparison of passenger density and number of organisms per 5 litres in corresponding observations on the air of railway carriages on the six railways concerned.

	Central London		Bakerloo		City and South London			
Passenger density in compartment	40.0	46.0	60.0	60.0	88.0	64.0		
No. of organisms in 5 litres	27.0 (at 20° C.)	15.0 (at 37° C.)	37.0 (at 20° C.)	21.0 (at 37° C.)	49.6 (at 20° C.)	20.0 (at 37° C.)		
	Piccadilly and Brompton		Hampstead and Highgate		Metropolitan District		Total railway carriages	
Passenger density in compartment	46.0	51.0	55.0	52.0	87.0	87.0	62.0	58.0
No. of organisms in 5 litres	41.0 (at 20° C.)	27.0 (at 37° C.)	41.0 (at 20° C.)	24.0 (at 37° C.)	31.0 (at 20° C.)	46.0 (at 37° C.)	40.0 (at 20° C.)	23.0 (at 37° C.)

demonstrate the relationship of passenger density and the bacterial content of the carriage air on each of the six railways.

In the case of the mesophil group there appears to be a general tendency for the bacterial and passenger curves to run parallel, except on the Piccadilly Railway, the number of organisms per 5 litres of air rising and falling with the increase and decrease of passenger density. This parallelism becomes more evident on the psychrophil side, especially on the Metropolitan District and South London Railways, but again in the case of the Piccadilly Railway and

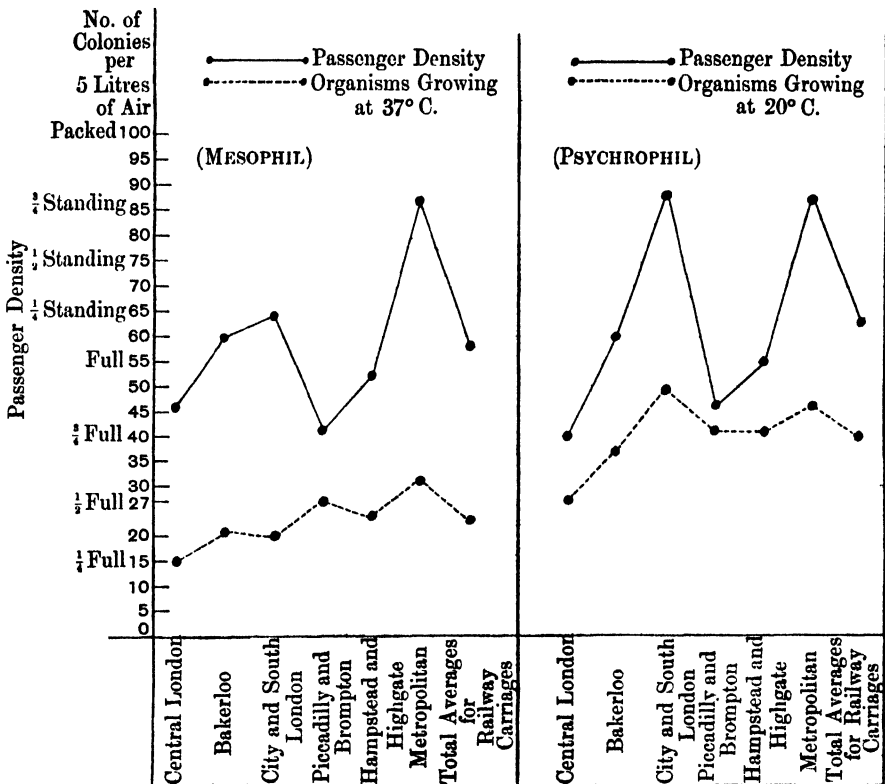


Chart I. Relationship of passenger density and bacterial air content—as shown by the mean of total observations made on corresponding dates.

to a less degree the Hampstead Railway, the lower mean of the passenger rates is associated with a relatively high average bacterial content.

The results of the individual observations taken consecutively under approximately the same conditions of passenger density for the two bacterial groups, show on Chart II a fairly close parallel agreement, though at different levels, between the mesophil curves of four of the railways, viz. Metropolitan District, Central London, and most marked for the South London and Hampstead and Highgate Railways, where the bacterial content of carriage air falls with the fall in passenger rate for each line.

In the case of the Piccadilly and Bakerloo Railways, however, there is not

the same conformity, the bacterial content showing a sustained rise for the one and an irregular rise and subsequent fall for the other railway, in spite of a mutual progressive decline of the passenger rate of both lines.

Similarly, on the psychrophil side of Chart II the fall in bacterial content and passenger density run parallel except on the Piccadilly and Hampstead Railways, both of which show a rising bacterial content with a falling passenger rate.

Though difficult to explain satisfactorily, discrepancies of the two temperature groups, especially in the case of the exceptions mentioned, notably the Piccadilly Railway, are to be partly accounted for by changes in passenger density occurring while the samples for the respective temperature groups of organisms were being taken on the lines concerned.

The mesophil group of organisms may be taken generally to include those derived from a human or animal source and does not embrace the large proportion of moulds which develop at the lower temperature. Broadly speaking, whilst the number of mesophil bacteria would seem to vary as the passenger density, this parallelism is apparently more marked in the case of the psychrophil group.

The comparisons of the various observations concerned, however, cannot be too closely driven on account of the differing phases of ventilation and movements both of train and passengers during the course of taking a single sample of 5 litres, a process occupying about eight minutes. In this time there occurred stoppages at stations, opening and closing of carriage doors, exit and entrance of passengers creating considerable changes in the currents of air circulating through the compartment and likely to affect the numbers of floating organisms. In addition to these temporary alterations, factors common to each particular line have to be borne in mind as influencing air movement, such as the position of the doors and the number and position of windows which may be open or closed. In the case of the Metropolitan District Railway carriages, where doors open at the sides of the carriages either in the middle or at the end, there is less encouragement for a clear passage of air through the compartment than in the case of the other lines, where many of the carriages have end-doors permitting of free current, and frequently it may happen that one end is left open throughout the journey.

A further factor, affecting the results of observations on the Metropolitan District Railway, is the type of ventilating window in use, particularly in the older carriages running on the Inner Circle. Of two types still used, one drops open on release of spring catches placed centrally or at each side, the other has to be raised, and kept open by supporting hooks. In either case the simultaneous use of both hands is required to open the window. This action few people are able or will trouble to carry out, consequently the window remains shut. The central double spring catch is more easily worked but is often out of order or the window frame refuses to drop. In all the other railways the ventilating frame is readily opened by depressing a ring

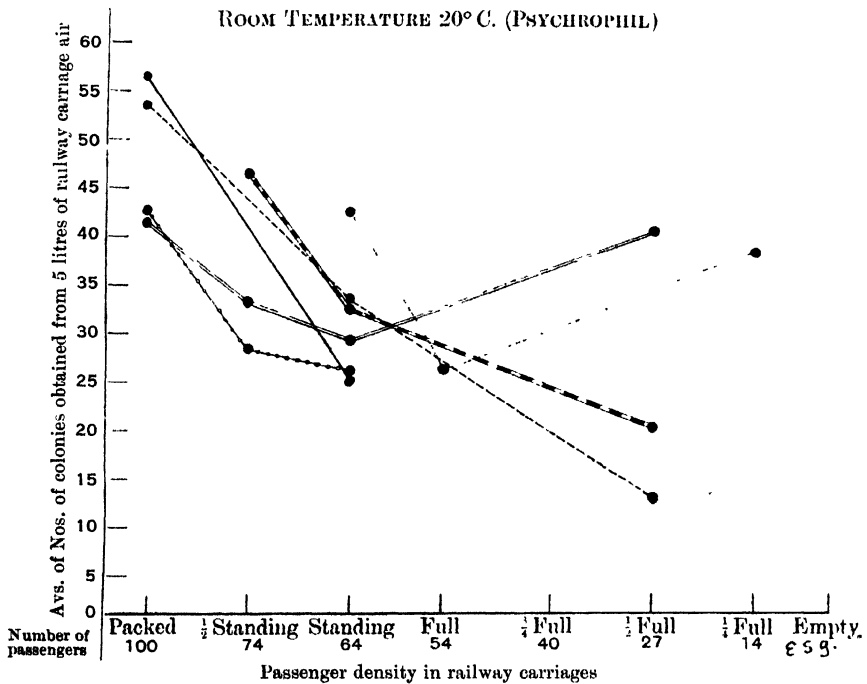
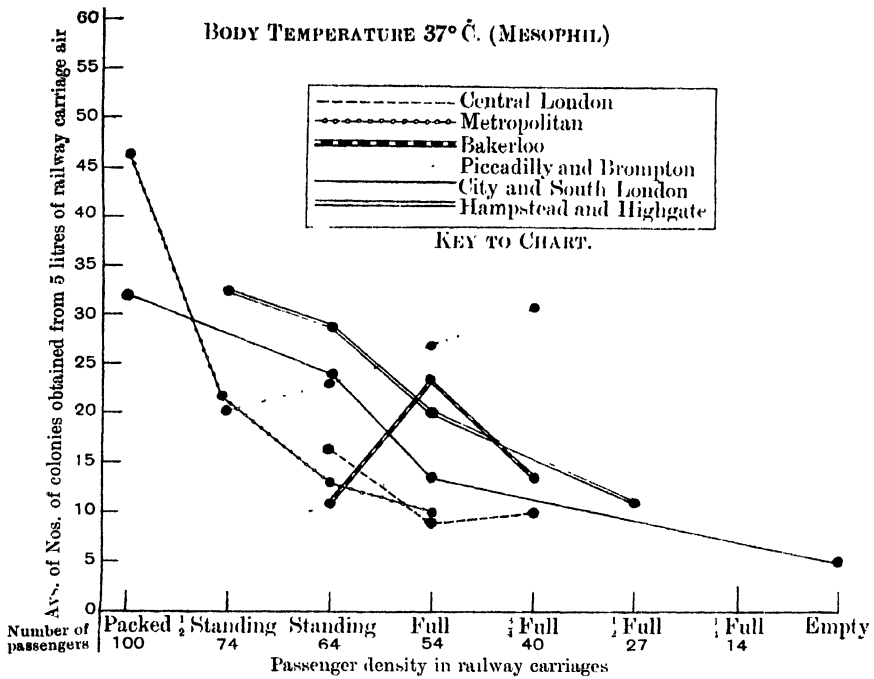


Chart II. Charts of individual observations taken consecutively and under approximately corresponding conditions of passenger density for each of the temperature groups and showing relations between passenger density in moving trains and number of organisms growing at body temperature and room temperature.

attached to the central catch and the window falls forward at once. From the greater simplicity of this type of window it follows that there is better prospect of ventilation than can be obtained on the Inner Circle, on which there would seem to be a tendency for the carriages to carry on the vitiated air from station to station.

There are, therefore, infinite possibilities of constant change of atmospheric conditions arising from fluctuating currents and passenger movement which may influence bacteriological results of individual observations, but by taking the averages of a number of results it would appear, from the charts and figures given, that a certain degree of uniformity and correlation between a series of observations bearing on bacterial content and passenger density can be secured.

With reference to the systems of ventilation employed and the means relied upon for renovating the air in the different Tube Railways, it may be stated here that the method in vogue on the Central London Railway, of the introduction of air by pumping engines combined with dependence on piston action of the trains in motion and an extracting fan at Liverpool Street Station, is probably more efficacious than the methods of ventilation used on the other railways. The Bakerloo, Piccadilly and Brompton, and Hampstead and Highgate depend largely on extraction fans and use only pumping engines at some of the change stations. The piston action of trains plays a part on these lines, and on the City and South London Railway it would appear to be the sole means of effecting change of air. In the case of the Metropolitan District Railway, whose stations are all open to the outside air, the piston action would for this reason be inhibited to a considerable extent. This fact and the absence of any pumping or extraction means, together with overcrowding, and on the Inner Circle the unsatisfactory type of window frame already mentioned, may possibly account for the high figures of observations obtained on the Inner Circle of the Metropolitan Railway and City and South London Railway.

In the report on the New York subways great importance is attached to the piston action of trains^(5g) moving through tunnels, especially non-stop express, in creating ventilation currents and change of air, to the extent of superseding the need of blow holes to the surface and rendering unnecessary the operation of mechanical devices such as fans. The superiority of train movement over fan power for the ventilation of underground railways is also emphasised in a report (1910) on the Hudson River Tubes⁽¹⁰⁾, which states: "The velocity and directions of the air is governed almost entirely by train movements and not by the fans... It is therefore evident that the function of the fans is only to take the air pushed to them by the trains or to deliver fresh air where the trains can push or draw it through the stations or tunnels, and this fact should mainly govern the locations for other fans and air ducts."

CORRIGENDUM

JOURNAL OF HYGIENE, Vol. xxii., p. 141, l. 8.

For 'quotient' read 'dividend'

VI. AN "INDEX OF BACTERIAL POLLUTION" OF RAILWAY CARRIAGE AIR.

For the purpose of estimating a suggested "index of pollution," observations derived from samples taken consecutively and under as nearly identical conditions of passenger density as possible for each of the two temperature groups have only been utilised. This involves the omission of certain individual observations figuring in the total results given on p. 130 and with which there was no corresponding sample in one or the other temperature group. By using the figure for the bacterial content as the divisor and that of the corresponding passenger density as the quotient, the ratio of the one to the other is obtained, giving the "index of bacterial air pollution" in possibly truer proportion for each railway than by consideration of bacterial content alone, irrespective of passenger density.

A comparison of the ratios of the one to the other shows that the index of pollution for each of the six railways varies in the mesophil and psychrophil groups and in the relative positions occupied by some of the railways in the two groups.

Moreover, the degrees of pollution of carriage air, viewed from the standard of bacterial content alone, undergo considerable rearrangement in the order of precedence (*vide* p. 130), when passenger density is taken into account and the ratios of the two are estimated as the "index of pollution." This rearrangement most affects the positions of the Metropolitan District and City and South London Railways in the psychrophil group, where they become respectively 1st and 2nd instead of 5th and 6th in order of purity. Conversely the Central London drops from 1st to 4th place whilst the positions of the other

MESOPHIL GROUP.

Railway		Average of number of organisms in 5 litres of carriage air growing at 37° C.	Average of corresponding passenger density	Ratio of bacterial content to passenger density
1. City and South London	(3rd)*	20	64	1 to 3.2
2. Central London	(1st)	15	46	1 „ 3
3. Bakerloo	(2nd)	21	60	1 „ 2.9
4. Metropolitan District	(6th)	31	87	1 „ 2.8
5. Hampstead and Highgate	(5th)	24	52	1 „ 2.2
6. Piccadilly and Brompton	(4th)	27	51	1 „ 1.9
Average for all railways		23	60	1 „ 2.6

PSYCHROPHIL GROUP.

		Do. at 20° C.		
1. Metropolitan District	(5th)	46	87	1 to 1.9
2. City and South London	(6th)	49.6	88	1 „ 1.76
3. Bakerloo	(2nd)	37	60	1 „ 1.6
4. Central London	(1st)	27	40	1 „ 1.5
5. Hampstead and Highgate	(4th)	41	55	1 „ 1.4
6. Piccadilly and Brompton	(4th)	41	46	1 „ 1.1
Average for all railways		40	62.6	1 „ 1.5

* Bracketed figures denote positions in order of air purity based on the averages of all observations on bacterial content alone; *v.* p. 8.

three railways are less affected, the Bakerloo falling from 2nd to 3rd, the Hampstead and Piccadilly Railways each dropping from 4th to 5th and 6th on the list.

In the mesophil group the changes in order are less marked; the Metropolitan District and South London Railways each gain two places, the latter displacing the Central London from the 1st place, the Piccadilly falling from 4th to 6th, changing places with the Metropolitan, whilst the Bakerloo and Hampstead Railways remain in their respective positions of 3rd and 5th.

From the evidence provided by the ratio of bacterial content to passenger density, reckoned as the "index of pollution," it would appear that the increase of the two does not occur in strict linear relationship—whereas with a rising passenger rate and overcrowding, the total bacterial content is undoubtedly increased, the increase in bacterial pollution of carriage air is not in direct proportion to the high passenger rate, but is relatively less per passenger than with a lower passenger density.

The observations on which the above ratios are based are however too few and their numerical relationships too close to afford sufficiently reliable data for drawing any but the most tentative conclusions.

Moreover, any such possible conclusions call for further reservation from the presence of additional factors already alluded to, which necessarily influence the bacterial content of subway carriage air and cannot be estimated, namely, dust disturbance created by air currents and passenger movements, and the unknown amount of residual bacterial impurity retained by and possibly peculiar to the compartment itself.

VII. SPECIES OF ORGANISMS FOUND IN THE AIR OF THE TUBE RAILWAYS.

(With reference in particular to the group of Moulds.)

Species of organisms found in Tube Railway air. The attempt at identification of the many varieties of organisms obtained in plate culture from samples of railway and platform air proved an extremely difficult and laborious task, the results of which can only be regarded in a large proportion as approximate.

The main object of the search has been, if possible, to find evidence of organisms pathogenic to man; for this reason, and in order to restrict the dimensions of the work, no detailed examination has been made of the cultural growth of colonies obtained in the control tests of the outside air. Such a comparison would without doubt have been of considerable interest and importance but appeared to be impracticable and outside the scope of the general purpose of the investigation.

As no facilities were available for animal experiments, any attempt at establishing the pathogenicity of a particular species was out of the question. Generally speaking, except in a few instances, the evidence provided by sub-culture tests failed to show the presence of organisms known to be pathogenic.

Such failure, which is indeed to be expected, attended the results of both Andrewes' and Graham-Smith's investigations. Attention was particularly paid to the possible presence of so common an organism as *Bacillus coli communis*, contained, of course, in horse dung and therefore likely to be conveyed everywhere by particles of dust. In no case, however, could it be identified.

There are many pathogenic organisms which will not long survive outside the contact of the human body, being unable to withstand the effect of desiccation and changes of temperature. Again, for successful cultivation under the most favourable circumstances, such delicate organisms require special media, *e.g.* blood serum or blood agar, for their growth, and even if these were employed the chances of their discovery among the numerous and more hardy varieties of air-borne organisms is infinitesimal.

[As pointed out to me by Mr J. H. Coste, F.I.C., the influence, too, of electrical discharge, whether by direct local action, radiation, or electrophoresis on the survival of organisms in the Underground Railways is quite unknown. Indeed the field of research offered by such consideration might lead to some explanation of the comparatively low degree of microbic pollution apparently prevailing in the air of the carriages and platforms.

The results of the examination of sewer air by Andrewes⁽¹¹⁾ and Horrocks⁽¹²⁾ have shown a remarkable degree of freedom from microbic pollution, possibly to be accounted for by the adherence of organisms to the moist surfaces and by the fluid stream flowing in a confined channel acting as a germ trap. With the substitution of electrical for fluid attraction, it is conceivable, on expert authority¹, that the deposition of germ-laden dust particles in railway air may be favoured by electrostatic force, such as is associated with the high potential conductor rails along the course of the Tube Railways, leading to a sedimentation and trapping of matter in suspension.]

Among the species approximately identified are certain organisms which have been located as existing saprophytically and, so far as is known, harmlessly in the mouth and nasal passages and about the skin of the body. The proportion of these organisms in railway air is roughly estimated at about 20 per cent.

Carnelly, Haldane and Anderson⁽¹³⁾ have demonstrated that bacteria are not given off in the *ordinary* respiration of healthy persons, and that the micro-organisms derived from the skin and clothes of persons actually present in a room are few in comparison with those attached to particles of dust, for which a room acts in the nature of a trap. The now well-known fact was also proved by Hesse⁽¹⁴⁾ that when a room is left quiet, micro-organisms settle out in a few hours leaving the air comparatively free. But with the disturbance of dust, as by stamping or shuffling the feet, the bacterial content of the air is enormously increased, although ordinary movements of many persons in a room, as the three observers above-mentioned have shown, are not sufficient to produce any marked change in the numbers of organisms.

¹ Prof. A. O. Rankine, D.Sc.

It is perhaps not too much to assume that, when harmless saprophytes are present in and recoverable from the air of Tube Railways, under existing crowded conditions of travel, there is every possibility that the pathogenic and more sensitive varieties of organisms may be conveyed from one passenger to another in the minute droplets expelled in the process of coughing, sneezing, laughing and loud talking. Experiments undertaken by Trillat and Mailein (15) have shown the important influence of moisture on the growth of organisms and have proved that whilst dryness of the air on the one hand is detrimental to their survival, with increasing atmospheric humidity on the other, conditions become favourable to the maintenance and development of bacteria and probably more particularly of those whose optimum temperature is that of the body. Hence in the railway carriage, a confined and often crowded space, the greater the humidity, the higher is the bacterial content of the air likely to be, and consequently the better the chance of the conveyance and survival of pathogenic organisms from passenger to passenger.

The correlation of the bacterial content with passenger density, which has already been mentioned and displayed on Charts I and II, has particular bearing in the case of the Metropolitan District Railway (Inner Circle) and City and South London Railway.

For the differentiation of the various species of organisms derived from the samples of railway air, subcultures were planted on agar-agar, gelatin and potato, in broth and litmus milk; occasional use was also made of certain sugar fermentation tests. Approximate recognition of the species depended on the response to these tests, the colour and character of growth, also on staining reactions of film preparations, and appearances in hanging drop as regards shape, arrangement and motility.

No attempt was made to obtain quantitative results for the different species by examining every colony on each plate; such a task would have been well-nigh impossible. Therefore a selection only was relied upon to yield approximate information as to what organisms were present. In all, over 250 original colonies from the gelatin and agar-agar plates of railway air samples were submitted to subculture tests. In addition six samples of 10 litres of railway air were specially examined, three for *B. coli communis*, and three for anaerobic organisms, without revealing evidence of either.

It has not been thought necessary to group the species according to the individual railways, but it is sufficient rather to summarise the various species for all the Tube Railways generally.

The main object of identification being to discover the presence of pathogenic organisms, *i.e.* those whose optimum temperature is that of the human body, the mesophilic group, attention has not been paid to the classification of the psychrophilic group or those particular organisms which prefer room temperature. Such an omission may be regarded as unscientific and one to be deprecated, but it was realised *ab initio* that so comprehensive and detailed an investigation was beyond the real scope of this survey.

In the New York investigations Soper frankly states that the determination of the various species of organism in subway air was regarded as impracticable and likely to yield no result of value, and the demonstration of pathogenic bacteria was held beyond the possibility of bacteriological technique^(5 d).

Interesting observations^(5 e) were however made on the viability of the pneumococcus by exposure of pneumonic sputum to the air of the New York subways. It was found that the organism still retained its virulence in dried sputum after 23 days' exposure, whereas in sunlight, as other observers had shown, it was killed in four hours.

M. H. Gordon⁽⁴⁾, in his report on the air of the House of Commons in 1906, states: "The majority of bacteria found in air are harmless and their individual significance, even if a name can be attached to them, conjectural, or at most botanical," and again, "The kinds of bacteria of most significance in air are primarily those capable of causing disease in man, and especially those capable of producing this effect when inhaled. Such micro-organisms, however, even when present in air are difficult to detect. Although of the greatest significance when found, the detection of specific pathogenic micro-organisms in air is so uncertain in the present stage of bacteriology that failure to find them by the methods at present available, even after careful search, does not necessarily imply their absence."

Nevertheless, the results of the detailed efforts to determine the identity of the various organisms present in the Tube air of the Central London Railway recorded by Andrewes⁽¹⁾ in his Bacteriological Report of 1902 afforded a study worthy of emulation, despite his failure to detect any true pathogenic organisms other than saprophytes of the human body.

On reference to bacteriological literature but little help was provided by the ordinary text-book, which deals almost entirely with disease-bearing organisms. But by recourse to such compilations as Sternberg's *Manual of Bacteriology*⁽¹⁶⁾ and Chester's *Manual of Determinative Bacteriology*⁽¹⁷⁾ it has been possible to obtain an approach to approximate classification and identification, but in a certain number it proved impossible to name the organism from records available.

Classification of micro-organisms obtained from the air of London Tube Railways

(1) Group of Coccus.

	No of times found	Source from which it was originally described
<i>Micrococcus albus liquefaciens</i>	10	Normal nasal mucus
" <i>flavus</i>	9	Air and water
" <i>candicans</i>	7	Air, water, milk, urine, etc.
" <i>candidus</i>	6	Water
" <i>lactericus</i>	4	From the human mouth
" <i>salivarius</i>	4	Saliva of man
" <i>nivalis</i>	4	Air
<i>Staphylococcus pyogenes albus</i>	3	Surface of body and deeper parts of skin
<i>Micrococcus citreus</i>	3	Water
" <i>simplex</i>	3	Water
" <i>albus</i>	3	Water and beer
" <i>cumulatus</i>	3	Nasal mucus in man

	No. of times found	Source from which it was originally described
<i>Micrococcus versicolor</i>	3	Air
„ <i>cereus</i>	2	Human abscess
„ <i>aurantiacus</i>	2	Air and water
„ <i>subflavus</i>	6	Nasal mucus
„ <i>aureus</i>	2	Air
„ <i>aquatilis</i>	2	Water
„ <i>magnus</i>	2	Air
„ <i>roseus</i>	2	Air
„ <i>cinnabareus</i>	1	Air and water
„ <i>conglomeratus</i>	1	Air and dust
„ <i>coronatus</i>	1	Air
„ <i>luteus</i>	1	Air and water
„ <i>aerius</i>	1	Milk
„ <i>albicans tardissimus</i>	3	Human secretion

(2) Group of *Bacillus* and *Bacterium*.

(a) Motile.

<i>Bacillus mesentericus vulgaris</i>	9	Widely distributed—dust, etc.
„ „ <i>fuscus</i>	6	Widely distributed—dust, etc.
„ <i>plicatus</i>	3	Milk
„ <i>subtilis</i>	4	Dust, etc.
„ <i>stellatus</i>	1	Milk
„ <i>fluoresceus crassus</i>	1	Air and water
„ <i>aurantiacus</i>	1	Water
„ <i>tenuis</i>	1	Milk
„ <i>solitarius</i>	1	Soil
„ <i>striatus flavus</i>	1	Water and surface of body

(b) Non-motile.

<i>Bacterium refractans</i>	3	Water
„ <i>nubilum</i>	2	Water
„ <i>acidum</i>	2	Milk
„ <i>vermiculosum</i>	1	Water
„ <i>rubidum</i>	1	Air
„ <i>xerosis</i>	1	Conjunctiva
„ <i>salivae</i>	1	Mouth
„ <i>aerophilum</i>	1	Air and water
„ <i>filiforme</i>	1	Water
„ <i>coccoidesum</i>	1	Milk
„ <i>ovale</i>	1	Water
„ <i>crassum</i>	1	Human sputum
„ <i>punctatum</i>	1	Milk

(3) Group of *Sarcina*.

<i>Sarcina lutea</i>	7	Air and mouth
„ <i>alba</i>	4	Air
„ <i>subflava</i>	4	Soil
„ <i>aurantiaca</i>	1	Air

(4) Group of *Streptothrix*.

In all 27 colonies in this group were sub-cultivated and placed provisionally among three varieties:

<i>Streptothrix albido</i>	Air
„ <i>chromogena</i>	Air, water and stomach contents
„ <i>foersteri</i>	Air and water

On account of the speciality of this group, sub-cultures were sent to the well-known mycologist, Prof. D. Pinoy, of the Pasteur Institute, Paris, for identification and classification. He very kindly examined the specimens, and his report states that the majority belong to the *Nocardia dissonviller*, which is a saprophyte, and appears only to have played a pathogenic rôle in

a case of ocular conjunctivitis reported by Landrien. Two varieties belong to another species not yet determined, but not pathogenic.

(5) Group of Moulds.

The proportion of moulds to bacterial colonies found:—

(1) in the open air control tests, out of a total of 222 colonies with 30 moulds, was as 1 to 7·5, or 13·5 per cent. of moulds. The highest proportion obtained (26·6 per cent.) was from a sample taken at the foot of the Duke of York's Column on a dull day with a moderate steady N.W. wind—the lowest (10·2 per cent.) was obtained in Charing Cross Gardens on a calm day with light southerly airs. The percentage obtained by Andrewes in his observations on the open air was 24·8, the highest (76·4 per cent.) being in a sample from Hyde Park.

(2) in the samples of air from all the railways, out of 1094 colonies with 136 moulds, the proportion was as 1 to 8·4, or 12·5 per cent. of moulds, the highest percentage figure obtained was 26 on the Metropolitan Railway (Inner Circle); the lowest 2·7 per cent. on the Central London Railway. On the various railways the proportion appears as follows:

	Proportion of moulds to bacterial colonies	Percentage of moulds
Metropolitan District	1 to 5·7	17·6
City and South London	1 „ 6·4	15·6
Piccadilly	1 „ 8·3	12·0
Hampstead	1 „ 8·5	11·7
Bakerloo	1 „ 8·8	11·2
Central London	1 „ 10·7	9·3

Andrewes, working on the Central London Railway only, obtained 93 moulds out of a total of 574 colonies or 16·2 per cent., the highest proportion in one sample being 44·4 per cent. and the lowest 1·8 per cent. [In the New York subway air the ratio of moulds to bacteria was 1 to 40, and usually the moulds were less numerous in the air of the subways than in the street air(5f).]

In the results obtained by Carnelly, Haldane and Anderson⁽¹⁸⁾ in Dundee in 1886, the ratio of bacteria to moulds stood in far higher proportion. The figures they give appear as follows:

	Proportion of moulds to bacteria
Outside air. Quiet places	1 to 2·5
„ Busy streets	1 „ 14·9
<i>Naturally ventilated schools:</i>	
Board schools	1 „ 131·8
Private schools	1 „ 30
<i>Mechanically ventilated schools:</i>	
General average	1 „ 28·5
Harris academy (Board school)	1 „ 31
Half-time school	1 „ 27
University College	1 „ 15·6
High School (cubic space much greater)	1 „ 4
<i>Dwelling-houses:</i>	
One-roomed	1 „ 49
Two-roomed	1 „ 20
Four- and more roomed	1 „ 21

They found that with increasing vitiation of the air the proportion of bacteria to moulds increased largely, and that this increase could be attributed to dust disturbances; of the two, bacteria settled out more speedily than the moulds as the air became quiet. It was recommended that in setting a standard of purity the ratio of bacteria to moulds should not exceed 30.

The conditions of atmospheric bacterial pollution prevailing in Underground Railways would seem to be in no way comparable to those obtaining in schools and dwelling-houses. The differences implied are largely due to the conflicting-phases of ventilation and the constant changes, produced by rapid movement of passengers and trains, inseparable from railway travel, and may serve to explain the lack of agreement to be found in the two series of observations which cannot fairly be placed side by side, as they do not afford equivalent grounds of comparison for the purpose of supplying a common standard of purity.

Classification of moulds. As in the case of identification of bacterial colonies so with the moulds, no attempt was made to subcultivate every mould appearing on the gelatin plates, but a selection was made and sent to Mr J. Ramsbottom, M.A., F.L.S., mycologist to the British Museum, South Kensington, who very courteously undertook their examination and has identified the following species¹:

(1) *Group—Phycomycetes.* *Mucor Mucedo* L., one of the commonest saprophytic fungi—principally on mouldy bread.

(2) *Group—Ascomycetes.*

(a) *Aspergillus (Sterigmatocystis) nidulans* (Eidam) Winter. Found on four occasions. Both stages of this fungus were obtained—the early conidial or *Aspergillus* stage and the later ascocarp (*Eurotium*) stage with budlike tufts. This fungus which was originally found on old bees'-nests has not previously been definitely recorded in this country.

(b) *Aspergillus (Sterigmatocystis) ochraceus* Wilhelm. What is probably this species was found in two cultures—sclerotia (hardening of the hyphal bed of the fungus) occurred which unlike the similar looking ascocarps of *A. nidulans* did not contain asci and spores. The fungus was first found on black bread—it also occurs on moist plants.

(c) *Aspergillus (Sterigmatocystis) niger* van Tieghem. Found on decaying plants, mouldy bread, etc.; also responsible for the pathological condition known as Aspergillosis—an infection by the mould attacking the ear passages occasionally, and more rarely the lungs; obtained twice in subculture.

(d) *Eurotium repens* (perfect stage of *Aspergillus repens*) (de Bary). Both conidial and ascospore stages—a common saprophyte—obtained once in subculture.

¹ Specimen cultures were subsequently sent to the National Collection of Type Cultures, Lister Institute.

(3) *Group—Hyphomycetes.*

**(a) Aspergillus fumigatus* Fresen.—occurs on decaying plants, mouldy bread, etc. Causes local inflammatory lesions in human beings especially in the ear (v. Siebenmann, *Die Schimmelmcykosen*, 1889), also responsible for pulmonary aspergillosis in Man, and found in the lungs, bronchial passages and ears of birds—obtained once in subculture.

(b) Penicillium glaucum Link. One of the commonest moulds. Recent work has shown that this is a composite species containing probably 80 to 100 micro-species—obtained nine times in subculture.

(c) Citromyces. A genus intermediate between *Penicillium* and *Aspergillus*; is a citric acid ferment and commonly found on fallen fruit—obtained twice in subculture.

(d) Acrostalagmus cinnabarinus Corda. A common saprophyte found on rotting potatoes, dung, etc.—obtained once in subculture.

(e) Cladosporium herbarum Link. Common saprophytic fungus.

(4) *Group of Yeasts* (5). The following were identified: *Monilia variabilis*, *Torula torulospora* (2). Two torulae were unnamed.

It may be pointed out that in the foregoing classifications, whilst it has been found impossible to place certain species of micro-organisms, for the reason that the works consulted give no clue to their identity and that there may be among them hitherto unnamed varieties of organisms, there is evidence of a proportion, amounting roughly to about 20 per cent. of the coccus, bacillus and sarcina groups, which although probably mere saprophytes and not recognised as pathogenic, were originally described as having been found in the mouth and nasal passages of human beings. Without laying too great stress on such sources of air contamination, the evidence is at any rate suggestive and significant of the possibility, in times of epidemic and in the crowded conditions of underground railway travel, of the conveyance of pathogenic organisms such as those of influenza, pneumonia and diphtheria from person to person.

Carnelly, Haldane and Anderson state⁽²⁰⁾:

“As regards the influence of the micro-organisms of air it seems probable that for persons in perfect health the great majority of organisms are harmless. The ciliated epithelium of the respiratory passages probably sweep them out as fast as they become entangled in the mucus with which it is bathed. Even those which have penetrated as far as the trachea and bronchial tubes are thus probably ultimately swallowed.

“The conditions are different, however, when there is even a slight catarrh of the respiratory passages. The bacteria in air are then probably a source of considerable danger. . . .”

* Castellani (19) in his Milroy Lectures (1920) on the Higher Fungi in relation to Human Pathology, states: “Aspergillomycosis of the ear is comparatively frequent.” He mentions cases apparently due to *A. fumigatus*, *A. niger*, *A. repens* and *A. nidulans*, all of which were identified in culture from Tube air.

A natural consequence of such catarrhal state, particularly under the influence of overcrowding during epidemics, is the liability to attack by influenza, acute bronchitis and broncho-pneumonia which, as the authors go on to emphasise, are largely responsible for the high mortality rates in measles and whooping-cough.

In summarising the chief points of this section it is incumbent to observe that our knowledge of the bacteriology of the air is still very incomplete and based perhaps on somewhat crude and unsatisfactory methods of investigation. Research on improved lines especially for the discovery of pathogenic organisms is still needed, including the examination of larger amounts of air and a greater number of samples than the work of this survey comprises. Much time, which it has been impossible to give, and additional assistance, which has not been available, would be required for carrying out particularly the more elaborate details of identification of the many species of organisms present in the air of the Electric Tube Railways.

SUMMARY AND CONCLUSIONS.

1. The bacterial content of the air of the Underground Railways, when the average of all results of the bacteriological investigations is taken, does not numerically compare unfavourably with the outside air of London.

2. The ratio of the number of organisms growing at room temperature appears to be about 14 for railway air to 10 outside air. For those growing at body temperature the ratio is considerably higher, namely 2 to 1 respectively. The mean per litre, for room temperature organisms, is about 9 in railway air, 6·3 in the outside air; for body temperature organisms 4·6 for railway air, 2·2 for outside air.

3. The bacterial content of platform air, except on the City and South London Railway, would appear to be higher than that of carriage air; the total mean for platform air being 52 and for carriage air 42·8 organisms per 5 litres, or a ratio of 16·4 and 13·5 respectively to 10 of the open air. The higher proportion in platform air is generally speaking to be accounted for by the greater amount of draught and dust disturbance.

4. The ratios of the total bacterial content of railway carriage air and carriage and platform air on the six lines to open air are estimated in the following proportions:

	Open air: 10.	
	Carriage air	Carriage and platform air
Central London	11·7	12
Bakerloo	12·6	14
Hampstead and Highgate	13	12·8
Piccadilly and Brompton	13	13·8
Metropolitan District (Inner Circle)	14·6	—
City and South London	15·7	15

5. Increase or decrease in passenger density is generally, but not invariably, associated with a rise or fall in the bacterial content of railway air

affecting both the room temperature group and body temperature group of organisms. The correlation is more evident on the Inner Circle of the Metropolitan District and the City and South London Railways.

6. With increasing passenger density and overcrowding the consequent rise in bacterial content of railway carriage air does not appear to follow in direct numerical proportion and may be relatively higher per passenger in a less crowded compartment, due possibly to the freer play of ventilation currents on dust particles offered by the clearer space.

7. The ratio of bacterial content to passenger density, though necessarily influenced by fluctuating air currents and movements of passengers in leaving and entering a compartment, is suggested as providing an "index of pollution" for carriage air and possibly affording a truer estimate of atmospheric bacterial pollution than if measured by bacterial content alone.

8. In comparison with the results obtained on the Central London Railway by Andrewes in 1902 the atmosphere on that railway in 1920 would appear to show a satisfactory state of bacterial purity, with a lower bacterial content than on other underground railways, notably the Metropolitan District and City and South London Railways, where the higher content may be attributable to overcrowding and unevenly controlled carriage ventilation.

9. In view of the rise in bacterial content of railway air consequent on dust disturbance by air and train movements, measures to bring about the deposition and removal of dust from carriages, platforms and running track are likely to prove beneficial by reducing the amount of floating bacterial impurity.

10. In no instance were pathogenic organisms specifically proved to be present other than certain of the moulds, *e.g.* *Aspergillus niger* and *fumigatus*. The apparently high proportion of micro-organisms suggested as emanating from the mouth and nasal passages of human beings indicates the possibility that crowding of passengers, with the existing ventilating arrangements for certain of the carriages and tubes, may be prejudicial in epidemic times to the public health by increasing the risk of transference of pathogenic organisms from passenger to passenger.

Post scriptum. June, 1923.

Three years have elapsed since the completion of the work upon which the above report was based.

During this period improvements in the system of ventilation and of tunnel-cleansing, as well as in carriage construction, have been steadily progressive. The latter has replaced to some extent the older type of travelling compartment. Overcrowding, however, during rush hours, and the occasional use of the earlier type of carriage, referred to on pp. 138 and 140, have not yet been eliminated. Happily there has been no serious return of influenza in epidemic form.

Cordial thanks and indebtedness remain to be expressed to the authorities of the Electric Underground Railways for ready facilities afforded to the Collection of Air Samples; to Mr J. H. Coste, F.I.C., for use of his helpful suggestions incorporated on pp. 143 and 144; also to Mr J. Ramsbottom, of the British Museum, for his valuable work in the identification of moulds, similarly to Prof. D. Pinoy of the Pasteur Institute, Paris; and, finally, to Mr F. E. Fry and Mr E. S. Glass for the latter's skilful draughtmanship of diagrams and charts, and for their joint help in the laboratory as well as in the arduous work of collecting the air samples.

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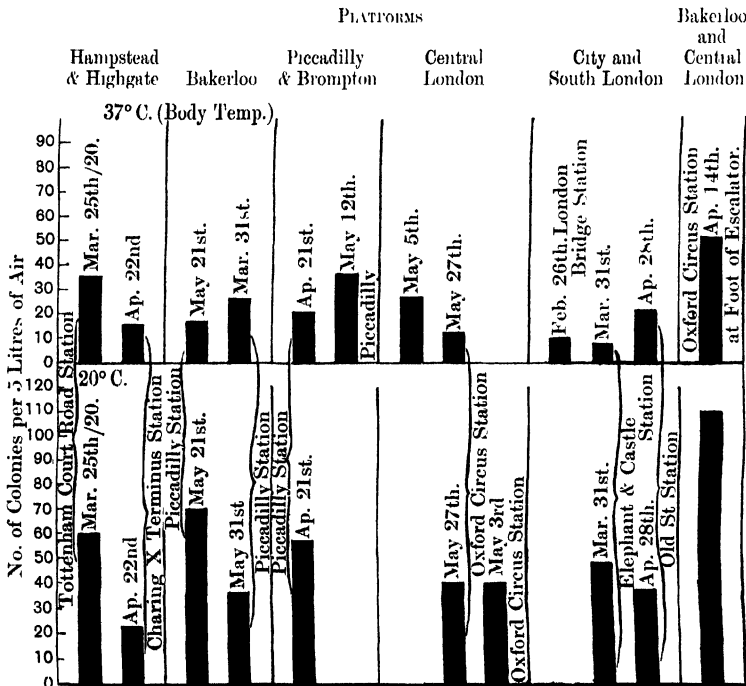
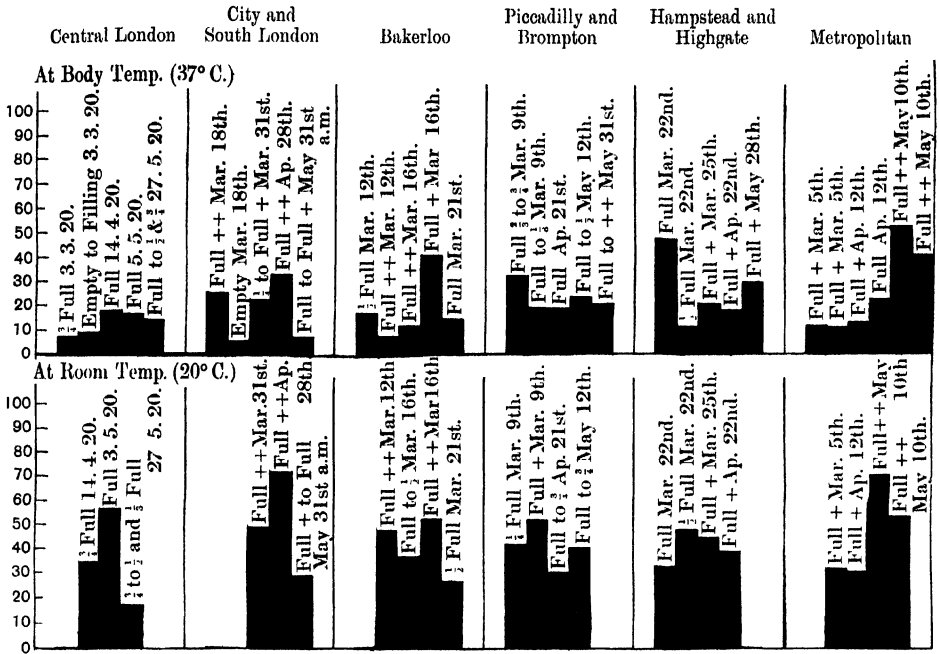
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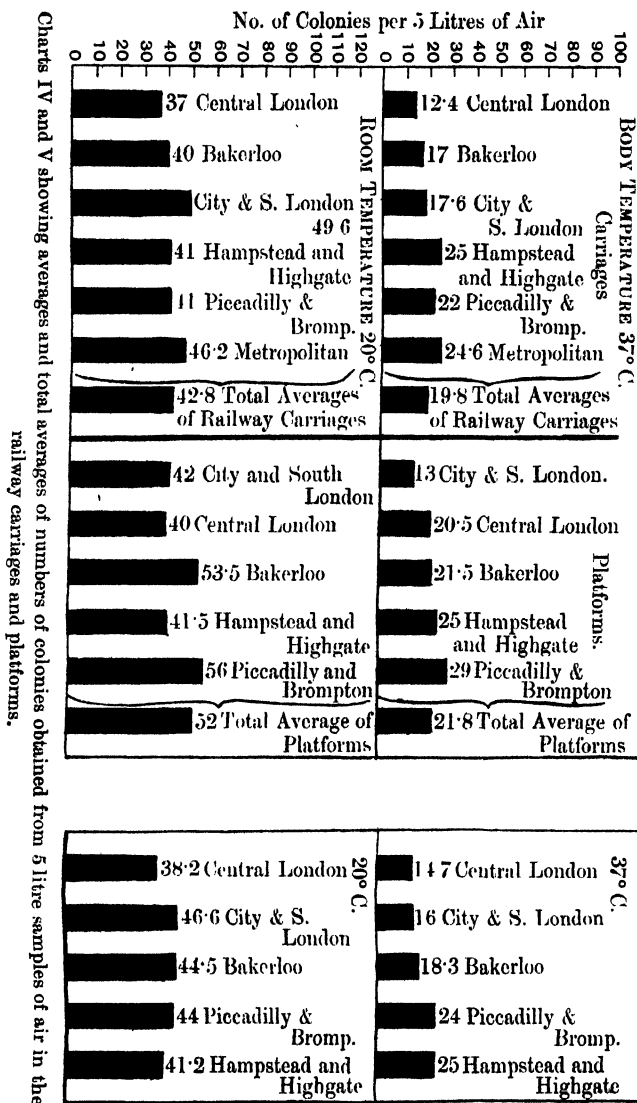
Comparative Block Charts are attached showing:

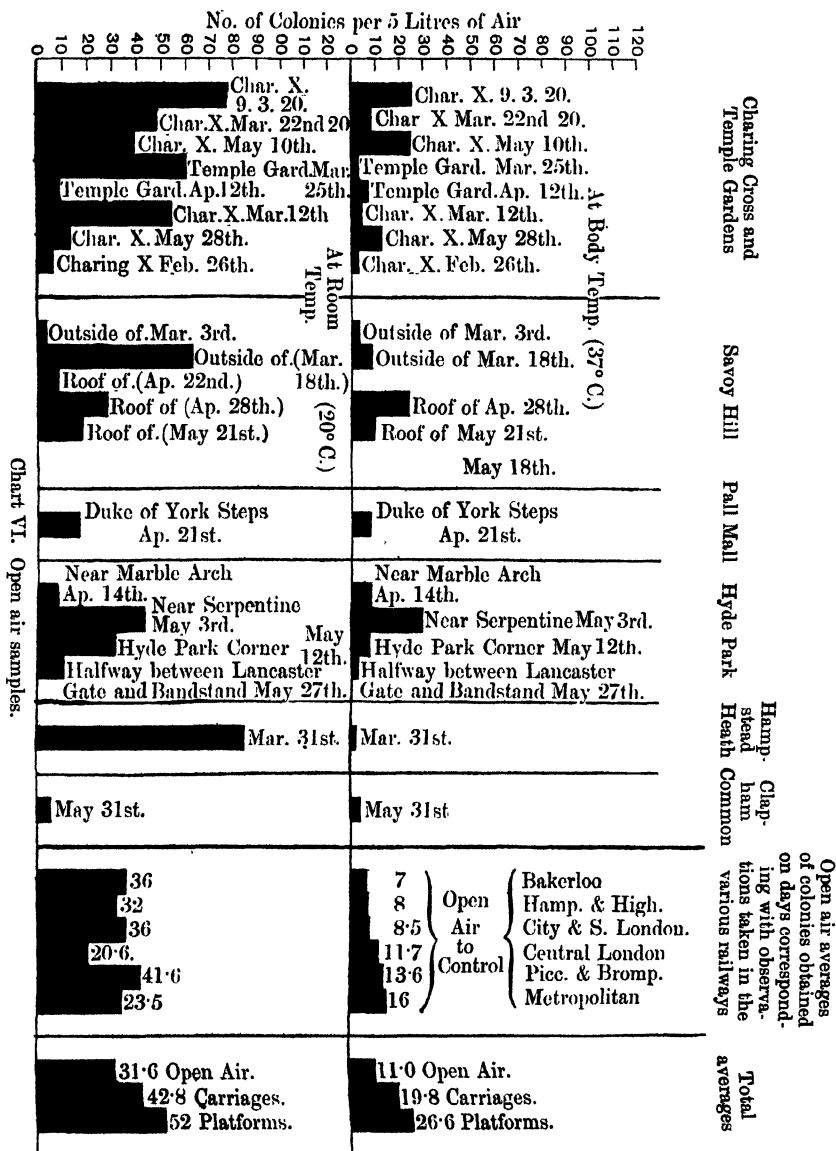
III. Number of colonies obtained from each 5 litre sample cultivated at body temperature and room temperature from carriage air (A) and platform air (B) on each railway.

IV. Averages of total observations on carriage and platform air of each railway.

V. Results of 5 litre samples taken in the open air and total averages of III A and B and IV







SPONTANEOUS AGGLUTINATION OF THE CHOLERA VIBRIO IN RELATION TO VARIABILITY

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SPONTANEOUS agglutination is a not uncommon occurrence, especially in the case of organisms of the enteric and diphtheria group, where it causes some difficulty in serological diagnosis. Many investigations have been made to clear up the principles of this phenomenon. Recently Arkwright (1921) has thrown much light on this problem. He was able to isolate from old pure cultures of dysentery, typhoid, paratyphoid and enteritidis (Gaertner) bacilli, two variants which he named the "R" (rough) and "S" (smooth). The "R" variant agglutinates spontaneously in physiological saline and in broth, while the "S" variant forms good emulsions in both. Specific immune sera prepared for these two variants show only slight cross-agglutination. Arkwright regards the two forms as potentially inherent in most of the individuals in the young culture, which may become segregated in different individuals in old cultures. "Rough" varieties have been observed also by Zoeller (1922) in *B. dysenteriae* Shiga and by Schütze (1921) in *B. paratyphosus* B.

Recently De Kruif (1921, 1922*a*, 1922*b*) has reported similar variation in *B. leptisepticum*. From spontaneous pneumonia of rabbits he isolated cultures which he was able to separate into two biotypes. The granular "G" type grows as clumps in broth, yields slightly irregular translucent non-fluorescent colonies on serum-agar, agglutinates easily at 37° C. and 55° C. with immune serum, and is only slightly virulent. The type "D" yields a diffuse turbidity in broth and presents smooth fluorescent colonies on serum-agar. In contact with immune serum it flocculates well at 55° C. but poorly or not at all at 37° C., and is highly virulent. The degree of cross-agglutination between the two types was examined. Serum prepared with the "D" form agglutinated both forms equally (1 in 2000) while that made with the "G" form, had a much lower titre (1 in 50 to 1 in 200) for "D" than for "G" (1 in 1000). The type "G" arose from the parent "D" by mutation.

Mellon (1922) has found in a pleomorphic diphtheroid bacillus two morphological phases of growth: a diplococcoid phase requiring a temperature of 20° C. forming stable emulsions in sodium chloride and Tyrode's solution; and a bacillary phase requiring a temperature of 37° C. and agglutinating spontaneously in both solutions.

A temporary spontaneous agglutinability of cholera-vibrios was observed by Friedberger and Luerssen (1905) in very young freshly isolated cultures; after 18–24 hours' growth at 37° C. this sensitiveness to salt disappeared.

Hamburger observed that cholera-vibrios grown for a long time in immune serum showed the phenomenon of spontaneous agglutination. This character was, however, lost after repeated passages on agar.

Baerthlein (1912) also made a number of observations on variation of the cholera-vibrio, especially on the two well-known variants which are distinguished by forming (1) transparent and (2) yellow and slightly opaque colonies. He found that the serological differences were very slight and he did not describe a spontaneously agglutinable form.

It was with the purpose of searching for similar variants of the cholera-vibrio that the present investigation was undertaken. Two old laboratory strains of vibrio (1) "Konia," a haemolytic strain and (2) "Kolle," non-haemolytic, were inoculated into broth tubes and incubated at 37° C. for 24 hours. The broth tubes were then kept in the dark at room temperature. Subcultured on nutrient agar after 15 days, the "Konia" strain showed two varieties of colony similar to the two types named by Arkwright the "R" and "S." Even after four months, the "Kolle" strain showed no tendency to split off a "rough" variety.

MORPHOLOGY AND BIOLOGY OF THE TWO TYPES OF "KONIA" STRAIN.

The colonies of the "S" type on nutrient agar are circular, with regular, well-defined margins, and, when examined with a low power of the microscope, appear very finely granular. The colonies of the "R" type are larger than the "S" colonies, are flat and thin, and when examined under the low power, they are very granular and have a jagged margin. The "R" form, in ordinary nutrient broth or peptone water, produces a deposit at the bottom of the tube, leaving the supernatant liquid clear. The "S" form produces general turbidity. A pellicle is formed by both types. The precipitation in cultures of the "R" type is no doubt due to the salt content of the medium, since the "R" form produces general turbidity without any deposit if cultured on broth or peptone water diluted with distilled water to one-half or one-quarter of the usual strength; and this is also the case if the medium is prepared with less salt. As regards motility, fermentation of carbohydrates, indol formation and proteolysis, the two types are identical. The haemotoxin-production of the two types was practically the same as is shown by the following experiment:

72 hours' growth of the "R" and "S" types on ordinary broth were filtered through a Berkefeld candle. Different dilutions of the filtrates were prepared with normal saline solution. To 1 c.c. of each dilution, 1 c.c. of a 2 per cent. emulsion of sheep's red blood corpuscles was added. The tubes were kept in the water-bath at 37° C. for two hours and then in the ice chest till next morning.

Result. Haemolysis occurred in practically identical dilutions of the two filtrates, i.e. in 1 in 50 but not in 1 in 100.

Agglutination of Cholera Vibrio

DEGREE OF AGGLUTINABILITY BY SALTS.

Table 1 shows the agglutinability of the three strains, "Konia," "S" strain and "R" strain, by calcium chloride and sodium chloride.

Table 1.

		NaCl				CaCl ₂										D.W.
		$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	
"Konia" strain		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"S" type		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"R" "		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		$\frac{M}{2}, \frac{M}{4}, \frac{M}{8}, \text{etc.} = \frac{\text{Molar}}{2}, \frac{\text{Molar}}{4}, \frac{\text{Molar}}{8}$														
		$\frac{M}{2} \text{ NaCl} = 2.9 \text{ per cent.} \quad \frac{M}{2} \text{ CaCl}_2 = 5.5 \text{ per cent.} \quad \text{D.W.} = \text{distilled water.}$														

Liefmann (1913) described a method which he advocated for differentiating bacterial species by flocculating emulsions with strong solutions of magnesium sulphate. He found that true cholera-vibrios were as a rule completely salted out in 80 per cent. saturation, whereas cholera-like strains almost invariably failed to show precipitation. Greig (1913) and Gildemeister and Günther (1919) repeated and extended these observations. While confirming the general results, they pointed out that exceptions were not infrequent.

Emulsions of the original strain "Konia" and its two variants were tested with strong solutions of MgSO_4 and it was found that whereas "Konia" and the "S" form were flocculated in 100 per cent., 90 per cent. and slightly in 80 per cent. of saturated MgSO_4 , the "R" type was flocculated in all dilutions examined, *i.e.* 100 per cent. to 10 per cent.

Porges and Prantschoff (1906) stated that if an emulsion of spontaneously agglutinating cholera vibrios was heated to 80° C. it was rendered stable, but that its agglutinability by specific serum was not impaired as occurred in the case of an emulsion of *B. typhosus*.

This procedure was tried with the "R" variant, but it was found that the agglutination by salts remained practically the same after heating.

Acid agglutination. Michaelis (1911) found that many species of bacteria were agglutinated by acids and that the agglutination for each species was at its optimum in a particular degree of acidity (*i.e.* of hydrogen-ion concentration). In the coli-typhoid group, he determined the special agglutination optima for *B. typhosus* and *B. paratyphosus* B and recommended this test as a method for the identification of these micro-organisms. Beniasch (1912), working under Michaelis, performed many experiments on the agglutination

of bacteria by acids. He came to the conclusion that the acid agglutination optimum was specific for the bacterial species and that even subgroups of the same species could in some instances be differentiated by this means.

Buffer solutions were prepared and the acid agglutination limits of the "Konia" strain, "S" type and "R" type were tested, by the method of Beniasch.

Result. The optimum agglutination of "S" occurred in a solution of higher acidity than that required by "Konia," while the agglutination of "R" took place over a wider range, with its centre in a lower acidity than the optimum for "Konia."

The agglutination optima for the emulsions were as follows:

"Konia"	[H]	1.1×10^{-3}
"S"	"	2.2×10^{-3}
"R"	"	between 2.8×10^{-4} and 1.1×10^{-3}

According to Beniasch:

V. cholerae	[H]	0.7×10^{-4}
V. metschnikovi	"	2.8×10^{-4}

IMMUNITY REACTIONS.

Separate antisera were prepared against the "Konia" strain, the "S" type and the "R" type. Rabbits received intravenous inoculations of heat-killed 24 hour cultures (60° C. for 30 minutes). Three inoculations at 5-day intervals were given, and one week after the last inoculation the animals were bled. The sera were preserved in 0.5 per cent. carbolic acid.

Method of agglutination. The macroscopic method was used. The emulsions were always prepared in distilled water and the serum was diluted with saline solution (0.42 per cent. NaCl). The agglutination was carried out in an incubator at 37° C. for four hours.

Table 2.

Dilution of serum	"Konia" serum tested against			"S" type serum tested against			"R" type serum tested against		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/50	+++	+++	+	+++	+++	++	+	+	+++
1/100	+++	+++	+	+++	+++	++	+	+	+++
1/200	+++	+++	+	+++	+++	++	-	-	+++
1/400	+++	+++	+	+++	+++	+	-	-	+++
1/800	+++	+++	+	+++	+++	+	-	-	+++
1/1600	++	++	(+)	+++	+++	-	-	-	++
1/3200	++	++	-	++	++	-	-	-	(+)
1/6400	-	-	-	++	++	-	-	-	-
1/12800	-	-	-	(+)	+	-	-	-	-
1/25600	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-

+++ = complete agglutination.
++ = good agglutination.

+ = distinct agglutination.
(+) = very slight agglutination.

These agglutination tests show that there is only slight cross-agglutination between the "S" and "R" varieties. The "S" type behaved like the parent "Konia" strain.

Absorption tests. These tests were carried out by mixing 1.5 c.c. of serum (diluted to 1/25 in 0.42 per cent. salt solution) with 1.5 c.c. of a strong emulsion of the culture in distilled water. This emulsion was made by adding the growth of one agar slope to 0.5 c.c. of water. After incubation at 37° C. for two hours, the mixture was put in the ice chest till the next day and then centrifuged. The supernatant fluid was examined for its agglutinating power in the way already described.

[illegible][illegible][illegible]

The results of the absorption experiments show that "Konia" and "S" sera lost their agglutinins for both these strains when absorbed with the "Konia" and "S" type strains, but not when absorbed with the "R" type strain.

The "R" serum lost its agglutinins when absorbed with the "R" strain but not when absorbed with either "Konia" or "S" strain.

Complement fixation: The serological relations shown to exist between the three varieties were also confirmed by complement fixation experiments.

Technique: The antigen was prepared by emulsifying a 24 hour agar slope culture in 10 c.c. of physiological saline solution (killed by heating at 58° C. for 30 minutes). 0.5 c.c. of the diluted serum + 0.5 c.c. antigen + 0.5 c.c. of fresh guinea-pig serum (diluted 1 : 10) were placed in the water bath for 40 minutes. Then 0.5 c.c. of a 5 per cent. emulsion of sheep's red blood corpuscles + 0.5 c.c. amboceptor (4 m.h.d.) were added. After 30 minutes in the water bath, the tubes were placed in the ice chest and the result read after four hours.

Table 6.

Dilution of serum	"Konia" serum with antigens			"S" type serum with antigens			"R" type serum with antigens		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/50	+	+	+	+	+	+	+	+	+
1/100	+	+	+	+	+	+	+	+	+
1/200	+	+	t.h.	+	+	+	t.h.	t.h.	+
1/400	+	+	t.h.	+	+	+	—	—	+
1/800	+	+	—	+	+	—	—	—	+
1/1600	+	+	—	+	+	—	—	—	t.h.
1/3200	+	+	—	+	+	—	—	—	—
1/6400	t.h.	t.h.	—	+	+	—	—	—	—
1/12800	—	—	—	+	+	—	—	—	—
1/25600	—	—	—	+	+	—	—	—	—
Control serum	—	—	—	—	—	—	—	—	—
Control antigen	—	—	—	—	—	—	—	—	—

+= complete inhibition.

— = no inhibition.

t.h. = trace haemolysis.

PATHOGENICITY.

The original "Konia" strain was of a very low virulence, but, in view of de Kruif's results with the two types of *B. leptosepticum*, an experiment was made in order to detect any difference in pathogenicity between the two variants.

Guinea-pigs of about equal weights were inoculated intraperitoneally with 1/20, 1/10, 1/5, 1/2 and 1 of a 24 hours' agar slope culture of both types.

Table 7.

	"S" type	"R" type
1/20 Agar Slope	Survived	Survived
1/10 "	Survived	Survived
1/5 "	Death	Survived
1/2 "	Death	Death
1 "	Death	Death

This experiment was repeated with the same result, from which it would appear that the "S" type is more pathogenic than the "R" type.

RESISTANCE.

It was interesting to see if any difference existed between the two types in their resistance to chemical disinfectants. Two methods of testing this point were used.

Inhibition of growth. To six tubes, each containing 4 c.c. of sterile broth, decreasing quantities of carbolic acid were added. The tubes were then made up with broth to 5 c.c. The inoculation was carried out as follows: 24-hour agar cultures were emulsified each in 2 c.c. physiological saline solution and after being brought to the same opacity, the emulsions were further diluted 50 times. 0.1 c.c. of this dilution was added to the tubes. The tubes were placed in the incubator at 37° C. for 7 days. The results were recorded after 1, 3, 5, and 7 days. Both the "S" and the "R" type multiplied and caused turbidity in 1/1600 carbolic acid but not in 1/800.

Death of organisms. Emulsions were prepared as in the previous experiment. Three drops of the emulsion were added to 5 c.c. of 0.5 per cent. carbolic acid. Directly and after 5, 10, 15, 30 and 60 minutes two loopfuls were inoculated into broth tubes which were incubated and the result recorded as in the preceding experiment.

Result: Both types were viable after 5 but not after 10 minutes.

These experiments show that the two types have practically the same resisting power to the disinfectant used.

The resistance to heat was tested by exposing emulsions to a temperature of 45° C. for varying periods.

Technique. One loopful of a 24 hour agar culture was emulsified in 5 c.c. distilled water. The tubes were put in the water bath at 45° C. and after 5, 10, 15, 20, 30, 40, 50 and 60 minutes one loopful was inoculated into broth.

Result: The "S" type could be subcultured after 40 and the "R" type after 50 minutes but not after longer periods, though the control tubes without carbolic were viable after 60 minutes.

This experiment, which was repeated many times, showed that the "R" variant is to a slight degree more resistant to heat than the "S" variant.

CONCLUSIONS.

1. Of two strains of *Vibrio cholerae* tested, one (a haemolytic strain) yielded two variants "S" and "R" resembling those described by Arkwright as occurring in the colon-typhoid group.

2. The "S" variant forms a stable emulsion in physiological saline and causes general turbidity in broth and peptone cultures. The "R" variant agglutinates spontaneously in physiological saline solution. In broth culture it forms a deposit, leaving the supernatant fluid clear. Emulsions in weaker saline solutions are stable, so that agglutination tests can be carried out.

3. The two variants differ in the following characters:

- (a) cultural characters on agar,
- (b) acid agglutination optima,
- (c) agglutination with specific sera, absorption of agglutinins and complement fixation,
- (d) pathogenicity,
- (e) resistance to heat.

4. They are identical as regards:

- (a) motility,
- (b) fermentation of carbohydrates,
- (c) formation of indol,
- (d) proteolysis,
- (e) haemotoxin production.

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A REPORT ON THE VENTILATION OF SCHOOLS

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(With 1 Graph.)

“HEALTH is more affected by atmospheric conditions than by any other influence. From the moment the new born child utters its first cry, until life finally ceases, he is directly and immediately dependent on the atmosphere he breathes for continued existence. Records of sickness and of mortality clearly show that the nearer atmospheric conditions are to those of the open country, the better is the health of the population, and that the more these conditions are modified owing to urbanisation and industrialisation, the more pronounced is the ill-effect upon general health” (1).

The vast importance and the far-reaching effects of this statement are at present only partially realised, and reluctantly admitted, but each year the medical statistician brings forward accurate and uniform findings, the significant deductions from which it is imperative to consider and utilise. Recent work on the epidemiology of phthisis by Collis and Greenwood⁽¹⁾ has shown that the factory, by confinement of the workers in monotonously ventilated rooms, and by causing general fatigue, reduces the resistance of the operative to those sources of infection to which he is exposed at home. These authors maintain that, in the light of this, “it is not through any special intensive measures of campaigning against the tubercle bacillus, not even by the segregation of the actively tuberculous does there seem any real hope of salvation.” But rather the ideal is to improve the conditions of the home and the factory.

Furthermore, Leonard Hill⁽²⁾ has shown that there is a considerable correlation between variations not only of the sense of well-being, but of actual prevalence of sickness, and the degree of *stagnation* of the air; and the whole group of respiratory affections (“colds,” catarrhs, sore throats, bronchitis, pneumonia, measles, cerebro-spinal fever, and whooping cough) are in their spread largely influenced by the moist heated atmospheres of indoor life.

This being the case, it is self-evident that the adequate ventilation, and efficient general hygiene of the home, factory, and school (for the school in child life corresponds to the factory of the adult) are most powerful influences in the prevention of disease. And, because prevention is surely the best economy, these measures must not be unwillingly considered, inadequately taken up or totally rejected. As Sir George Newman writes, “The Public Health is not only a matter of the postponement of mortality and the prevention of sickness, but of the positive side of health—the increase of vitality, capacity and efficiency of the human body....To secure this end, we must pay attention not only to actual ailments and diseases, but to the conditions making for a

maximum degree of personal health." And, surely, one of the most important of these conditions in the case of the child is the Hygiene of the School-room.

In the present report, the ventilation of four schools has been investigated: Schools A, B, C and D. The first two were taken as examples of the Plenum System, School C, a one-storey building of recent design (1913) was chosen to represent the most favourable conditions likely to be found in any elementary school with natural ventilation; and School D (built 1873) was the example of an older building without mechanical ventilation.

The methods of investigation are:

- (1) CO₂ estimation.
- (2) Readings of the kata-thermometer.
- (3) Relative number of organisms present.
- (4) Incidence of disease.
- (5) Personal test.

The reason for using each method, and the relative importance of the results will be dealt with under each heading; but it is convenient to mention, at this juncture, that owing to lack of time, some of the results obtained are inadequate in number. This is especially the case with the estimations of CO₂ and of the bacteria present in the air: but less time was purposely spent in these estimations, for, as will be seen, there is not very much importance attached to these results now, and consequently the general conclusions are not affected.

(1) CO₂ ESTIMATION.

The CO₂ present in the air was estimated by Pettenkofer's method, and the results are set out in Table 1 (at the end of this report). It will be seen that the schools with natural ventilation give approximately the same results as those where the Plenum system is in use.

Originally, attention was directed towards the gaseous condition of the atmosphere, and the air was deemed bad or good according as the proportion of CO₂ was more or less than a certain arbitrary standard—such as 0.1 per cent. (Pettenkofer), 0.13 per cent. (Carnelly, Anderson and Haldane). The advocates of such standards had to modify their views when it was demonstrated that air with large amounts of CO₂, amounts such as are never met with under ordinary conditions (*e.g.* 150 to 300 times the amount present in pure air) produced no ill-effects when breathed. It was then claimed that CO₂ was a useful measure of the poisonous emanations and respiratory impurities, but careful investigation has failed to establish the existence of such qualities.

The most recent view is that the chemical composition of occupied rooms is adequate for all the needs of respiration and that ill-effects are not dependent upon the balance of oxygen and carbon dioxide present. Therefore, not much attention is paid in this report to the results of CO₂ estimation. As Collis and Greenwood⁽¹⁾ say: "The fetish of a low CO₂ content must be replaced by the sound doctrine—long enough known instinctively to the people—that moving air, not a draught, but a breeze is health-giving."

(2) KATA-THERMOMETER READINGS.

Leonard Hill⁽²⁾ says, "the evaporative power of the atmosphere has a far-reaching effect not only on the comfort of the skin, but on the respiratory mechanism, the absorption of water from the gut and the renal secretion. The ceaseless variations in the rate of cooling, evaporation, absorption of radiant energy, as in outdoor conditions, relieves us from monotony, stimulates tone and metabolism." In pressing this point of view he urges that the whole question of ventilation depends not upon the supposed presence of injurious chemical products, but on *movement* of air of suitable temperature and humidity.

To the subject of the amount of movement required, Leonard Hill has devoted much research: he has designed an instrument to measure comparatively and approximately the rate of heat loss of the body under varying conditions. This instrument is the Kata-Thermometer (down-heat-measure): it is a large bulbéd spirit thermometer with a safety bulb at the top. In use, the bulb is dipped into hot water until the alcohol rises into the safety bulb, and then the instrument is dried and suspended in the air to be tested: the time taken to fall from 100° to 95° Fahr. (2½° above and below normal body temperature) is measured with a stop watch. The instrument is then used wet, *i.e.* with a silk mesh finger stall placed over the bulb, the excess water shaken off and the readings taken. Several readings (three or four) should be registered with each and the average worked out.

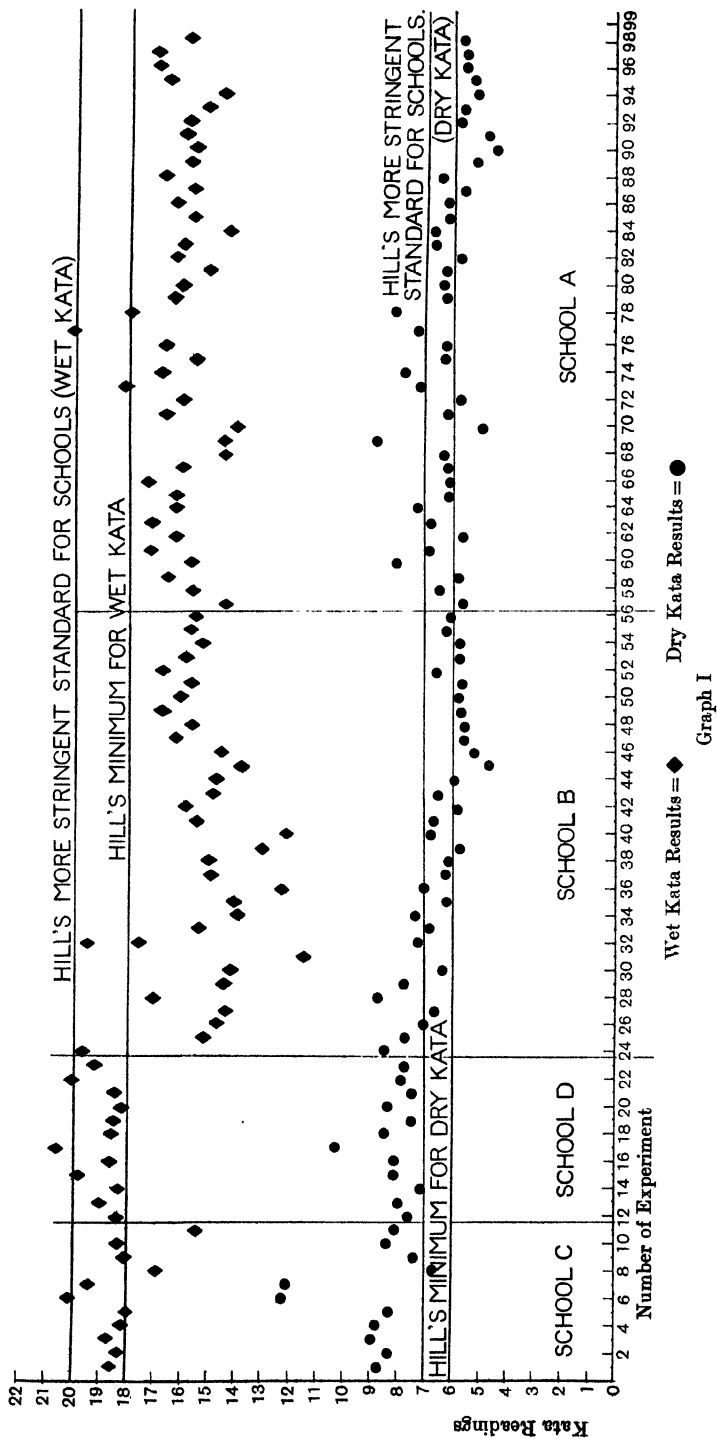
The dry bulb gives the cooling power by radiation and convection, the wet by radiation, convection and evaporation: to find this, each instrument possesses a factor (which has been evolved under specific conditions) and into this factor is divided the number of seconds which has been found by the experiment in question. This gives the cooling power in milli-calories per square centimetre per second: and the standards which Hill lays down as absolute minima are:

For dry kata the reading should not be less than 6.

For wet kata the reading should not be less than 18.

Preferably in schools, the dry reading should be over 7 and the wet over 20, because children, having a greater metabolism per unit of body surface than adults, need an atmosphere with not less, but a *greater* cooling power.

The results obtained in the four schools are shown in Table 2, and the readings are plotted in Graph I. It will be seen that Schools C and D show uniformly good readings: on only two occasions does the wet reading fall below 18, and this was caused by the windows in the particular rooms not being properly controlled. By altering the windows, the readings were brought above the minimum. In many of the experiments it will be noticed that the results for the dry kata were over 7 and for the wet exceeded 19, so that they nearly conformed to Hill's more stringent standard for schools. This is perhaps to be expected at School C, for that is a model building, situated in a very



favourable open position on the outskirts of the city: but it is indeed gratifying to find such results at School D, which is situated in the centre of the city, in one of the poorer districts, surrounded and enclosed by buildings on all sides.

On the contrary, the two schools with mechanical ventilation give far less favourable results: both at School A and School B it is the exception to find the wet reading over 18, and on many occasions the dry reading was below 6—in one experiment as low as 4.5. Moreover, it must be admitted that during the time these experiments were carried out, the wind was uniformly north-easterly, and the ventilation appeared from personal tests better than is the case in the more usual “muggy” type of English weather. Experiments 90–98 inclusive were taken on such a “muggy” day and show less favourable results: and further experiments will be carried out to ascertain if this difference is correct. On those occasions where the readings were above the minima laid down by Hill, there is a definite reason, and attention is drawn particularly to experiment 77 where readings of 7.3 for dry kata and 20.1 for wet kata were obtained, because the teacher, who stated that the children were falling asleep owing to the oppressive atmosphere, had opened the windows about ten minutes before the experiment was taken. The rooms next to it (experiments 75 and 76) had readings much lower, and these rooms had no windows open.

It may be objected that opening certain windows vitiates the working of the system, and that consequently certain experiments are not a true test: therefore, to meet this criticism, experiments 44–56 inclusive were carried out with the system working as its designers planned, all windows being closed. The results are uniformly worse than are obtained under the conditions which the teachers bring about by opening a few windows.

To show the importance of movement in the air so far as kata readings are concerned, experiments 29 and 32 may be cited: in the former, the wet kata reading was 14.5 with the class sitting still and 15.8 with the children waving their arms. Similarly in experiment 32, the reading was 19.5 when the girls were dancing, and only 17.6 when no dancing was in progress.

With the dry kata-thermometer, it is possible to measure the velocity of the air, and experiments were carried out to ascertain the velocity of the air as it entered the rooms from the shafts. The results are recorded in Table 3: it will be seen that even on the same day, the variations in the velocity had no definite relationship to the kata readings. Presumably, then, the velocity of the incoming air is never sufficient, as a general rule, to give such necessary movement as will allow good kata results.

(3) ORGANISMS.

The air of occupied rooms is known to contain bacteria, the number of which vary according to the degree of cleanliness and the amount of ventilation. All organisms are not pathogenic, and the estimation of the number present at any time is no certain indication of danger arising from infection.

Therefore not much importance is attached to this method of investigation. These experiments, recorded in Table 4, were carried out under conditions approximately the same (for merely marching the children out of the room would increase considerably the number of organisms falling per unit area), and it will be seen that the average for the two different types of school is approximately the same.

(4) THE INCIDENCE OF DISEASE.

Of very great importance is the comparative incidence of infectious disease, because it may be taken to show, in a definite way, the effects of different methods of ventilation. Unfortunately, it has been impossible in the schools under consideration to obtain any conclusive figures, but the general opinion of those who have worked for considerable periods in both types of school is that the sickness rate among staff and pupils alike is decidedly higher in the Plenum school. This is certainly in keeping with other observations, and the following illustrations of the importance of ventilation on the spread of disease may be cited.

"We find that the window-ventilated room at 67° Fahr. is characterised by materially lower incidence of respiratory disease than the fan-ventilated room at 69° Fahr....The window-ventilated room is somewhat more comfortable" (9).

Fan-ventilated rooms showed 18 per cent. more absences due to respiratory illness and 70 per cent. more respiratory illness among children in attendance, in spite of the fact that *per capita* floor space was greater in these than in the window-ventilated (9).

In an Australian troopship, three decks were well, and the fourth badly ventilated: the incidence of infective pharyngitis and epidemic catarrh was ten times greater among the troops on the badly ventilated deck (3).

Again, in 1914-15, the Canadian troops were camped in tents on Salisbury Plain under extreme conditions of wet and discomfort, but with excellent health. As soon as they moved into huts, catarrhs and sore throats spread rapidly, and several cases of cerebro-spinal fever occurred (2).

(5) THE PERSONAL TEST.

A. T. Nankivell, after a long series of experiments from May to December, 1915, in the four camps of the Dorset Training Area, found that the personal test was the best guide in ventilation, and quite as reliable as any of the orthodox chemical standards. Parkes and Kenwood⁽¹⁾ maintain that the sense impression of the state of the atmosphere "is probably one of the most reliable guides in judging the conditions of the atmosphere. It is the summing up by the individual's senses of everything that makes for the production of unhealthy conditions, whether this be temperature, excessive moisture or lack of movement in the air."

Certainly, the Plenum schools give an unpleasant sensation on entering the building, and the feelings of drowsiness, inertia and lassitude persist:

it is therefore not surprising that those who work in such schools should complain of the marked discomfort of the ventilation.

GENERAL CONCLUSIONS.

The general conclusions reached, then, seem to indicate decisively that the mechanical systems of ventilation, as they are worked in Schools A and B, are definitely unsatisfactory: excluding the considerations of the personal tests (for in that the individual may be alleged to be biased) the bare scientific results detailed above show that no other conclusion can legitimately be given.

It is easy to criticise adversely and to find fault, but it is vastly more difficult to formulate a balanced constructive criticism; and in attempting to find a solution, even a palliative remedy for the defects now patent, it is convenient to consider the ideals of ventilation. The desiderata are:

- (a) "Cool rather than hot."
- (b) "Dry rather than damp."
- (c) "Diverse in temperature in different parts and at different times rather than uniform and monotonous," and
- (d) "Moving rather than still" (5).

Or again, "Successful ventilation depends on the prevention of stagnation of the body heat on the one hand, and uncomfortable chilling of the body on the other. Not only should it prevent heat stagnation in schools and workshops, but it should stimulate the worker" (6).

RECOMMENDATIONS.

Palliative measures to improve the present systems at these schools include:

(1) *Installation of fans* for extraction and for remedying the stagnation of the air; and these fans must be so placed in relation to the Plenum inlets as to ensure complete ventilation of the room with suitable air movement at the working places. In addition, if desired, localised air circulation may be obtained by flat paddles or wafters. The construction and installation of any of these should be controlled experimentally by kata-thermometer readings, and one or two installed as a trial rather than the whole number fitted in immediately. For details of types of fans, optimum places in which to place them, and other particulars, it is very desirable to refer to the useful information in the Home Office Publication on the Ventilation of Factories and Workshops (7).

"The effect of a fan put to stir the air in a factory is shown by the following:

Dry kata	Wet kata	
4.0	15.0	Fan off
6.0	25.5	Fan on" (6).

(2) *Intervals of work.* During play-time, and after school, the air of the rooms could be greatly improved by throwing all windows and doors open and obtaining a through draught for a few minutes. As at present used, the Plenum system is only worked from about 8 a.m. to 4 p.m., Monday to Friday in-

clusive each week, and thus every evening from 4 p.m. (although evening classes are held in these schools) until the next morning, and all day on Saturday and Sunday, the air is unchanged and complete stagnation results. By opening windows even for a few minutes, much can be done, as is shown in the case of a cinema theatre where, by opening all doors in an interval, the CO_2 sank from 44 to $17\frac{1}{2}$ parts per 10,000 (6). At school during play-time and after school hours, the monitors in each class could be made responsible for opening and shutting all windows, and the head teachers agree that this could be done. Careful supervision, however, would have to be used, because in some schools the infants go out to play at different times from the older children, and this might create a difficulty; but it would not be of great importance if the whole Plenum system was put out of action for the duration of the combined play-time. As the Building Regulations for Elementary Schools lay down, "Adequate means for ventilating all rooms used for teaching must be provided, not only for admitting fresh air during use, but for flushing the rooms effectually during the intervals" (8).

Finally, the all-importance of ventilation must again be urged, for "health is more affected by atmospheric conditions than by any other influence" (1): as Hill (2) writes of the evils resulting from the indoor life under which conditions the majority of people are compelled to exist:

"Cramming in close rooms for competitive examinations makes pale weaklings of growing boys and girls. The confined life in office and workshop destroys good looks, weakens strength, lessens happiness and shortens life.... The streets of our cities are trod by hosts of men and women ugly of complexion, ill-grown, weaklings, withered and worn, incapable of strenuous muscular action at a time which should be their prime, developed in cunning, fear and sensitivity to pain by a mean struggle for existence in mean streets."

Preventive Medicine must strive continuously and vigorously to remove this terrible indictment, and so far as the School Medical Service is concerned, must hold high the ideal one day to be achieved: when the conditions of the school will materially help the child to grow strong enough to know something of the "joie de vivre," and when the conditions of the class-room will enable the teacher to understand the unfathomable wisdom of the philosophy that to all men "Labour is the joyful business of life."

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Table 1.

Estimation of CO₂ (Pettenkofer's method).

School	Date	Time	Room	No. of children	Artificial light or other contamination	Temp. ° Fahr.	% CO ₂	Dry kata	Wet kata
B	21. ii. 23	4.0	3	32	Nil	62	0.104	6.7	14.4
"	22. ii. 23	3.50	7	36	1 gas iron for 1 lesson	62	0.135	*	11.5
"	23. ii. 23	3.40	5	40	Nil	63	0.118	6.9	15.4
"	26. ii. 23	11.0	5	40	"	62	0.074	6.1	15.1
C	27. ii. 23	4.0	A	40	"	62	0.103	8.7	18.6
"	28. ii. 23	11.0	A	40	"	58	0.074	8.8	18.1
"	28. ii. 23	2.30	B	44	"	59	0.114	*	*
"	28. ii. 23	3.45	H	40	"	65	0.165	8.3	18.0
"	1. iii. 23	11.0	F	34	"	65	0.167	6.7	16.9
A	2. iii. 23	2.30	2	37	"	66	0.139	6.5	15.7
"	2. iii. 23	3.45	5	45	"	66	0.136	5.9	16.6
"	7. iii. 23	2.10	4	48	"	68	0.134	6.2	16.3
"	7. iii. 23	3.40	6	44	"	65	0.135	*	*

* Reading not taken.

Average CO₂ for School B, 0.12 %; School C, 0.12 %; School A, 0.14 %.Kata readings are shown in order to demonstrate complete absence of any relationship between CO₂ content and good or bad kata readings.

Table 2.

Kata-Thermometer readings.

No. of Exp. (1)	School (2)	Date (3)	Time (approx.) (4)	Room (5)	No. of children (6)	Temp. ° Fahr. (7)	Dry kata (8)	Wet kata (9)	Remarks (10)
1	C	27. ii. 23	4.0 p.m.	A	40	62	8.7	18.6	
2	"	"	4.15	B	45	64	8.3	18.3	
3	"	"	4.30	Hall	—	58	8.9	18.7	
4	"	28. ii. 23	11.0 a.m.	A	40	58	8.8	18.1	
5	"	"	3.45 p.m.	H	40	65	8.3	18.0	
6	"	"	4.0	F	36	58	12.2	20.1	
7	"	"	4.15	E	36	61	12.1	19.4	
8	"	1. iii. 23	11.0 a.m.	F	34	65	6.7	16.9	
9	"	"	11.30	D	35	63	7.4	18.1	
10	"	"	11.40	G	40	63	8.4	18.3	
11	"	"	11.55	I	34	65	8.1	15.4	
12	D	16. iii. 23	2.35 p.m.	B	24	61	7.6	18.4	
13	"	"	2.45	C	22	62	8.0	19.0	
14	"	"	2.55	D	35	65	7.1	18.3	
15	"	"	3.50	A	40	61	8.1	19.8	
16	"	"	3.15	E	35	56	8.1	18.6	
17	"	"	3.35	F	44	56	10.3	20.6	
18	"	20. iii. 23	3.40	B	24	61	8.5	18.6	
19	"	"	2.45	C	22	63	7.5	18.5	
20	"	"	2.55	D	35	62	8.4	18.2	
21	"	"	3.5	A	40	62	7.5	18.4	
22	"	"	3.20	E	35	64	7.9	20.1	
23	"	"	3.30	F	44	66	7.8	19.2	
24	B	21. ii. 23	3.45	Hall	—	57	8.5	19.7	
25	"	"	3.55	6	40	62	7.8	15.2	
26	"	"	4.10	11	38	61	7.0	14.7	
27	"	"	4.25	3	32	62	6.7	14.4	
28	"	22. ii. 23	3.50	Hall	—	58	8.8	17.0	
29	"	"	4.10	13	25	64	7.8	14.5	Wet kata 15.8 with children waving arms
30	"	"	4.20	15	46	60	6.4	14.2	
31	"	"	4.30	7	36	62	—	11.5	
32	"	23. ii. 23	3.40	Hall	—	56	7.3	19.5	Dancing in progress. Wet kata 17.6 with no dancing

Table 2—(continued)

No. of Exp. (1)	School (2)	Date (3)	Time (approx.) (4)	Room (5)	No. of children (6)	Temp. ° Fahr. (7)	Dry kata (8)	Wet kata (9)	Remarks (10)
33	B	23. ii. 23	3.50 p.m.	5	40	63	6.9	15.4	
34	"	"	4.5	7	36	65	7.4	14.0	
35	"	"	4.15	13	25	58	6.3	14.1	
36	"	"	4.25	15	46	60	7.1	12.4	
37	"	26. ii. 23	11.0 a.m.	3	40	60	6.3	15.0	Wet bulb therm. 54.5° F. Dry bulb therm. 55° F.
38	"	"	11.15	5	40	62	6.1	15.1	
39	"	"	11.25	14	37	66	5.7	13.1	
40	"	"	11.40	11	43	64	6.8	12.3	
41	B (inf.)	21. iii. 23	11.10	6	30	57	6.7	15.5	
42	"	"	11.30	Hall	45	62	5.8	15.9	
43	"	"	11.45	7	28	62	6.5	14.9	
44	"	23. iii. 23	2.20 p.m.	6	30	60	5.9	14.8	All windows throughout the school were closed on 23. iii. 23
45	"	"	2.35	7	29	64	4.6	13.9	
46	"	"	2.40	1	37	65	5.2	14.6	
47	"	"	2.50	2	34	63	5.5	16.3	
48	"	"	3.0	4	42	63	5.5	15.7	
49	"	"	3.5	7	28	64	5.7	16.8	
50	"	"	3.10	6	40	66	5.8	16.1	
51	B (mixed)	"	3.20	1	8	66	5.7	15.7	
52	"	"	3.25	13	26	59	6.6	16.8	Room 13 facing prevailing wind
53	"	"	3.45	15	52	66	5.8	15.9	
54	"	"	3.55	16	47	66	5.8	15.3	
55	"	"	4.5	10	39	64	6.3	15.8	Room 10 facing prevailing wind
56	"	"	4.15	3	38	64	6.1	15.6	
57	A (inf.)	2. iii. 23	2.30	1	39	66	5.6	14.5	Open fireplace
58	"	"	2.45	2	37	66	6.5	15.7	
59	"	"	3.30	5	45	60	5.9	16.6	
60	"	"	3.40	6	44	59	8.1	15.7	
61	"	"	3.50	8	28	61	6.9	17.2	
62	"	7. iii. 23	2.15	1	40	66	5.6	16.3	Open fire
63	"	"	2.45	2	36	65	6.9	17.2	
64	"	"	3.0	3	48	62	7.3	16.3	
65	"	"	3.10	4	48	68	6.2	16.3	
66	"	"	3.20	5	44	—	6.1	17.3	
67	"	"	3.50	Hall	—	63	6.2	16.0	
68	"	8. iii. 23	3.20	5	45	65	6.3	14.5	
69	"	"	3.35	3	48	67	8.8	14.5	
70	"	13. iii. 23	11.25 a.m.	5	45	66	4.9	14.1	
71	"	14. iii. 23	2.45 p.m.	4	46	70	6.2	16.7	
72	"	"	2.50	5	45	65	5.8	16.0	
73	"	"	3.0	7	42	62	7.2	18.1	Room 7 facing prevailing wind
74	A (mixed)	"	3.10	1	26	62	7.8	16.9	
75	"	"	3.45	4	42	63	6.3	15.5	
76	"	"	3.50	5	34	63	6.3	16.7	
77	"	"	3.55	6	59	61	7.3	20.1	Teacher had just opened window. Said "Children were falling asleep"
78	"	"	4.0	8	46	60	8.2	18.0	Room 8 facing prevailing wind
79	"	"	4.10	9	55	59	6.3	16.4	
80	"	"	4.15	13	34	59	6.4	16.1	
81	A (inf)	16. iii. 23	11.55 a.m.	2	34	65	6.3	15.1	
82	"	26. iii. 23	10.5	1	43	62	5.8	16.3	

Table 2—(continued)

No. of Exp. (1)	School (2)	Date (3)	Time (approx.) (4)	Room (5)	No. of children (6)	Temp. ° Fahr. (7)	Dry kata (8)	Wet kata (9)	Remarks (10)
83	A	26. ii. 23	10.25 a.m.	4	44	59	6.7	16.2	
84	"	"	10.35	7	24	62	6.7	14.4	
85	"	"	11.0	Hall	19	63	6.3	15.7	
86	"	"	11.25	2	35	62	6.3	16.4	
87	A (mixed)	"	11.35	1	40	63	5.7	15.7	
88	"	"	11.35	8	55	60	6.5	16.7	
89	"	"	11.50	3	38	65	5.2	15.8	
90	A (inf.)	27. iii. 23	2.20 p.m.	1	43	66	4.5	15.6	Open fire
91	"	"	2.35	2	36	64	4.8	16.0	
92	"	"	2.40	5	47	64	5.8	15.9	
93	"	"	2.55	7	16	63	5.7	15.2	
94	A (mixed)	"	3.5	1	—	68	5.2	14.6	
95	"	"	3.15	2	5	64	5.3	16.7	
96	"	"	3.30	6	50	66	5.6	17.1	
97	"	"	3.45	13	1	64	5.6	17.1	
98	"	"	3.50	16	45	64	5.7	15.9	

Table 3.

Velocity of air entering or leaving room by ducts.

School	Date	Time	Room	No. of children	Temp. ° Fahr.	Velocity (metres per second)	Dry kata	Wet kata
B	26. ii. 23	11.0 a.m.	3	40	60	0.951 (incoming air)	6.3	15.0
"	"	11.20	5	40	62	1.74 extraction shafts	6.1	15.1
"	"	11.40	14	37	66	0.423 " "	5.7	13.1
A	2. iii. 23	2.30 p.m.	1*	39	66	0.303 (incoming air)	5.6	14.5
"	"	2.45	2	37	66	1.47 " "	6.5	15.7
"	"	3.30	5	45	66	0.56 " "	5.9	16.6
"	"	3.40	6	44	59	1.85 " "	8.1	15.7
"	"	3.50	8	20	61	2.68 " "	6.9	17.2
"	7. iii. 23	2.15	1	40	66	0.203 " "	5.6	16.3
"	"	3.0	3	48	62	2.09 " "	7.3	16.3
"	"	3.10	4	48	68	1.22 " "	6.2	16.3
"	14. iii. 23	2.45	4	46	70	0.6 " "	6.2	16.7
"	"	2.55	5	45	65	1.47 " "	5.8	16.0
"	"	3.5	7†	42	62	1.52 " "	7.2	18.1
"	"	3.15	1	26	62	1.18 " "	7.8	16.9

* Room 1 has an open fireplace in addition to shaft for incoming air.

† Facing a strong wind on that day.

The velocity of the air entering the rooms from the shafts appears to show no relationship to the kata readings taken in different parts of the room at the level of the children.

Table 4.

Number of organisms.

(Calculated as the number falling per square foot per minute.)

School	Date	Time	Room	No. of children	Organisms	Remarks
B	27. ii. 23	3.50 p.m.	15	46	37	
"	"	4.0	7	36	24	
"	23. ii. 23	3.40	Hall	Drill class (30)	153	Dancing and drill going on
"	"	4.0	5	40	25	
C	27. ii. 23	4.0	A	40	23	
"	1. iii. 23	11.0 a.m.	F	34	22	
A	7. iii. 23	3.0 p.m.	3	48	19	
"	"	3.10	4	48	31	
"	"	3.40	6	44	31	
D	19. iii. 23	11.0 a.m.	B	24	38	
"	"	11.0	C	22	31	
"	"	11.10	D	35	29	
"	"	11.20	A	40	35	
"	"	11.20	E	35	67	Change of class. Movement during experiment
"	"	11.30	F	44	24	

Omitting the two experiments vitiated by movements, the average for Schools C and D is 29 and for Schools A and B, 28.

SOME ASPECTS OF MENINGOCOCCAL VIRULENCE

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED
OUT AT THE UNIVERSITY OF CAMBRIDGE FIELD LABORATORIES

By E. G. D. MURRAY.

CONTENTS.

	PAGE
I. Introduction	175
II. Conditions influencing the determination of the minimal lethal dose .	177
(a) Viability and purity of culture	178
(b) The health of the experimental animal	178
(c) The general influence of the nutrient medium	178
(d) The correct measurement of the dose of living cocci	180
(e) The strict correlation of dose to body weight of animal	180
(f) The influence of sub-culture and age	181
(g) Summary	183
III. The graduation of doses and the selection of the minimal lethal dose .	183
IV. The variation in virulence of freshly isolated strains	185
V. The fate of meningococci injected intraperitoneally into mice	186
VI. Interference with the activity of the leucocytes and the peritoneal folds .	190
VII. The raising of the virulence of the meningococcus by animal passage .	194
VIII. The raising of the virulence of the meningococcus <i>in vitro</i>	196
IX. The influence of desiccation on virulence	202
X. Discussion	203
XI. Conclusions	206
XII. References	207

I. INTRODUCTION.

"VIRULENCE" is an ill-defined term and its use frequently gives rise to disputes, which remain unresolved because the disputants have no common basis other than that the term applies to organisms which cause disease. Thus it is imperative that the writer clearly defines the meaning of the term virulence as used in this paper, that the issue may not be confused by the reader approaching the subject from a different point of view.

Since the term "pathogenicity" expresses the power possessed of microbes to cause disease and since pathogenic organisms exist which cannot of themselves actively invade the living tissues of another organism, virulence is not invariably a factor in pathogenicity; for the first limitation the writer places on the meaning of the term virulence is that it shall only apply to organisms capable themselves of actively invading the *living* tissues of the host. Not only is this capability to invade actively and to multiply in the living tissues of another organism very unevenly distributed amongst a relatively small number of bacteria, but its expression, as determined by the aggressive and

defensive machinations of the organisms concerned, is so varied that almost every case has to be studied as a particular example of a multiplicity of factors which are exceedingly difficult to analyse. These factors or "forces" to a large extent form the object of study in infection and immunity, and our present knowledge does not allow of a complete appreciation of their existence and significance.

From the writer's point of view, *Virulence is the resultant of the opposed systems of forces, exerted physiologically by both the parasite and its host, in the efforts of each to maintain its life and health.*

The intensity of the aggressive mechanisms of the parasite and the intensity of the defensive mechanisms of the host vary independently, but the algebraical sum of these two quantities, which must of necessity have opposite signs, expresses the success or failure of the attempted infection; that is to say, the parasite either survives or is destroyed—it is virulent or non-virulent. Generally speaking, it is not essential for the host to die; the success of the parasite is sufficiently complete if it can maintain its life so easily as to cause the disease to run its recognised clinical course.

But the need of the laboratory is not satisfied by this clinical manifestation, which could only be applied experimentally to animals subject to the disease it is desired to study, and then is limited by the powers of clinical observation, which are insufficient even in man.

Lacking a precise appreciation of the contributing factors, the measurement of virulence by a laboratory test must of necessity be a death struggle and its interpretation is not directly concerned with the mechanism whereby death is brought about; except in this far that it is essential for the investigator to be satisfied that the defeat of either protagonist is directly due to the other.

The virulence of a parasite is expressed, therefore, in terms of the death of one or other of the specified antagonists and a not unnatural bias has determined the indication of the result of this conflict to be Positive or Negative, according as the parasite or the host respectively conquers. For the same reason any process proper to the parasite, whether aggressive or defensive, is termed in this paper an "assailing factor" which means to say that it contributes to positive virulence.

The degree of virulence of bacteria is generally expressed in terms of the smallest dose administered in a living condition by a stated route, which constantly proves fatal to a chosen kind of experimental animal. Also it is customary to state that a given culture is non-virulent, or has lost its virulence for a particular experimental animal, when the experiment has resulted in the death of the parasite or, at least in the survival of the experimental animal.

Thus the purely relative significance of the term virulence requires a precise statement of the conditions of experiment, and for the purpose of this paper, the measure of virulence of the meningococcus is chosen to be the least amount of viable young culture, expressed in grammes, which, when injected

intraperitoneally at one time will cause the death of 20 gms. of mouse within 48 hours. When, in this paper, a culture is described as possessing "high virulence" or "low virulence," it is meant that the balance of interacting factors contributed by both host and parasite has favoured the parasite in the former case and the host in the latter.

II. CONDITIONS INFLUENCING THE DETERMINATION OF THE MINIMAL LETHAL DOSE.

The estimation of virulence in its simplest form, resolves itself into the problem of determining the Minimal Lethal Dose of the living bacterium for a specified animal. Whether this is easy or difficult, is determined by two general conditions which themselves are very complex:

1. Whether or not the bacterium under investigation causes natural disease in the available experimental animal.
2. The nature, degree and rapidity of alteration taking place in the bacterial culture under the conditions imposed by growth on artificial nutrient media.

Now, in relation to the first condition, there is no known experimental animal which naturally affords the conditions required by the meningococcus to reproduce the characteristic human disease. It is true that von Lingelsheim (1905), Flexner (1907) produced meningitis in monkeys, by the extreme procedure of sub-dural inoculation with meningococcal cultures and that Weinberg (1909) described a case of spontaneous meningitis in a chimpanzee due to an organism very closely resembling the meningococcus. Also, that a meningitis of horses, goats and sheep is known, which is caused by the *Diplococcus intracellularis equi* (Johne, 1896) of which the morphology and cultural characters differ very little from Weichselbaum's coccus and a clinical, epidemiological and pathological account of the disease of Borna would amply describe cerebro-spinal fever in man (Cadéac, 1914). Nevertheless, it is possible to inject large quantities of virulent meningococcus culture, freshly isolated from human cases, intravenously into horses without causing meningitis and Robertson-Milne (1906) failed to produce meningitis in goats by sub-dural inoculation with meningococcus culture.

Thus, although the usual experimental animals can be killed by dosing them with meningococcus or its products, it cannot be claimed that they are susceptible to that parasite in the same way that man is. Difficulties are to be anticipated therefore and relatively large doses may be expected to be necessary to establish the desired minimal lethal dose.

It is important to notice in this connection, considering the fact that the typical disease is a meningeal infection and the relatively large number of cases which recover without specific treatment, that the meningococcus must possess a relatively low grade of virulence.

A mere glance at the literature of meningococcal infections imposes an embarrassment of choice between the numerous and varied culture media ardently recommended, and almost every serious entrant into this field of

research introduces either a new medium or some modification of an old one. So marked is this feature that there is ample justification to anticipate difficulties arising in relation to the second condition expressed above; nor need there be any hesitation in attempting to add another item to the menu, since it is evident that the ideal medium has not yet been found.

For these reasons it comes about that the feeble and irregular killing power of the meningococcus is emphasised throughout the literature, and Dopter (1921, p. 425) summarises the situation, after describing how the virulence of a culture varies from day to day, by saying: "The minimal lethal dose is impossible to define." Horder and Gordon (1907), Gordon (1917) expressed the resistance of experimental animals in terms of the number of doses they could support administered at intervals of one hour, though in later work Gordon (1920) used single doses, but not without disappointment.

The writer claims to have probed some of the more important conditions it is essential to recognise and by so doing to have made it possible to establish a minimal lethal dose for the meningococcus. These conditions will now be briefly outlined.

(a) *Viability and purity of culture* are obviously essential conditions, but the necessity for control cultural and microscopical examinations requires emphasis, on account of the peculiarly sudden way in which a meningococcus may die under apparently favourable conditions. Failure of an experiment is occasionally adequately explained by reference to these controls. At the same time there is no evidence that the meningococci capable of surviving in artificial cultivation represent the sum of the viable cocci *in vivo*, therefore a colony count is not considered necessary, but it is required to prove that a sufficiently large proportion of cocci are still living.

(b) *The health of the experimental animal* is of primary importance as survival or death is the indicator used to read the result of the experiment. It is not possible to give too much attention to the breeding and care of experimental animals. Post mortem examination frequently reveals the cause of an anomalous result to be intercurrent disease and this applies particularly to mice which have been bought from a dealer.

(c) *The general influence of the nutrient medium* used is of considerable importance, but its exact degree is undetermined. The growth requirements of the meningococcus have been given a great deal of attention and this subject must be dealt with in a separate report, although certain features will be considered briefly here inasmuch as they bear directly on the subject. A variety of media have been used, such as Gordon's tryptic agar, Nicolle's M.M., peptone serum agar, etc., and all present intrinsic difficulties to attempted rigorous standardisation, therefore other methods have been devised to meet certain difficulties which will be described elsewhere. Fluid media have been avoided on account of the difficulty they present to accurate measurement of the quantity of growth used as a dose and the poor yield compared to growth on agar media.

The value of an agar medium for growing the organism is estimated by the average weight of dried growth obtained from unit area; this has been used as an index of uniformity in various batches, and a very large number of such comparisons have been made. With any medium there is an appreciable variation in the growth obtained in different subcultures of a given strain on the same batch of medium and this depends partly upon the surface moisture of the medium, which can be controlled to some extent by observing the precaution of having the medium and plates at the same temperature at the time of pouring, partly upon the depth of the medium, but more particularly upon the phase of growth of the organism which will be mentioned presently.

Not only do the amount and physical properties of the growth vary with the various kinds of medium, but there is complete disagreement between the yields of different batches of a given medium, made as ordinarily described; the extreme variation for a series of batches of tryptagar is as much as 25 per cent. on either side of the arithmetical mean yield of cocci per unit area of that medium. For this reason any technique by which animals are dosed in terms of agar-slopes, Roux-bottles, etc., alone will be beset by an enormous error which is still further multiplied when the variation in percentage of moisture is taken into account; it is calculated that the total error due to variation in growth by such a method of measuring doses of living meningococcus may easily amount to 100 per cent., which makes it quite impossible to compare any two experiments. It must be stated that very considerable variations in yield per unit area are not appreciated by simple inspection.

Even when the dose of living meningococcus is accurately weighed, thus eliminating the error due to variation in mass of growth per unit area, a considerable error is introduced by the variation in the percentage of dry coccus represented in the growth obtained on the ordinarily described media; the figures for a series of batches of tryptagar show a variation of 20 per cent. on one side and 13 per cent. on the other side of the arithmetical mean of the determined percentage content of dry cocci in the moist growth allowing of a possible error of 33 per cent. in dosing animals with living cocci.

As the variation in percentage of moisture on each side of the mean for a given batch of such a medium, is much less than the variation on either side of the mean for a number of different batches, it is evident that the method of making the medium does not supply a standard material.

The complicated ingredients required for bacteriological media to grow obligatory parasites, present considerable difficulties to the consistent realisation of the optimal conditions necessary to obtain uniformity of growth. But, so far as the meningococcus is concerned, the medium we have attempted to standardise has reduced the range of variation to a considerable extent; for, not only does the determined percentage of moisture exhibit the same arithmetical mean for a given batch as for a series of batches, but the figures show a variation of -5 per cent. on one side and $+7$ per cent. on the other side of the average determined percentage of dry cocci represented in the moist

growth, thus allowing a possible error, due to variation in moisture, of 12 per cent. in dosing animals with living cocci; and this error is further reduced to 7 per cent. by basing all calculations on the mean percentage of dry cocci. These results seem to indicate that further improvement is possible and it is evident that, in determining the minimal lethal dose of the living meningococcus, it is of primary importance to estimate the errors which the nutrient medium may introduce.

(d) *The correct measurement of the dose of living cocci* is a matter of difficulty, because the problem of estimating the proportion of viable cocci in a mass of growth has not been solved. It has been found by repeated sub-culture of the meningococcus at a 24 hour interval, that, on a medium giving the most luxuriant cultures, there is a wave of growth, indicated by a progressive increase in yield per unit area of medium followed by a sudden drop to minimal growth. The range of this fluctuation is usually in the region of + 20 per cent. and - 12 per cent. on the arithmetical mean yield for a good medium and is not appreciable to the eye, but on certain media the drop in growth can readily be perceived by inspection. This wave of growth appears to have a fairly regular period and in our experience the drop in the curve occurs every third day during the early part of a long series, but the interval becomes shorter as the sub-cultures are continued. Apart from any suggestion as to its immediate cause, this periodic rise and fall in the number of viable cocci contained in a given mass, is of profound importance with regard to the accurate measurement of dose in experiments depending upon the viability of the cocci. A virulent strain tested in the transition generation, might very easily give results showing an apparent loss of virulence, whereas in all probability a large proportion of the cocci injected were dead.

In dealing with very large masses of cocci, as is frequently necessary and desirable, it becomes important to take into account the specific volume of meningococcal growth; it has been determined by experiment that if this factor is taken as 1.0, it is well within the range of other experimental errors.

Still greater accuracy obtains if all doses are correlated in terms of the percentage of dry bacterial protoplasm, determined on an aliquot part of the actual growth used for any experiment.

(e) *The strict correlation of dose to body weight of animal* very materially influences the regularity of the result obtained: the inoculation of an animal with a known weight of culture amounts to diluting the virus with mouse, rat, etc., and it is quite as important to measure the amount of animal used as it is to know the amount of solvent required to dilute a standard solution to a desired degree.

The following method adopted by the writer facilitates this undertaking and depends upon the fact that if an emulsion is made so that the dose for 20 gms. of mouse is contained in 1 c.c., then the dose for a mouse of any weight will be as many 1/20th parts of a c.c. as the number of grammes the mouse weighs.

The dose is given in proportion to an arbitrarily chosen unit of animal (20 gms. of mouse, 100 gms. of rat, etc.) and, for this purpose, every animal is weighed to the nearest twentieth of a unit, which factor is determined by the error the physiological processes of the animal might introduce into the weighing and is proportional to the general error of experiment. These weighings take very little time and very soon become established as a routine procedure. The required dose for an animal of any weight is obtained thus: let the chosen unit of animal be denoted by U ; now dilute the substance it is desired to inject so that the dose for xU gms. of animal is contained in a volume V c.c., where x is any whole number. The volume of the dose of this dilution required for any animal less than xU gms. weight is given by the formula:

$$\text{Dose} = \frac{\text{Weight of Animal} \times d}{x \cdot U} \cdot V \text{ c.c.} \dots\dots\dots(1)$$

or more conveniently:

$$\text{Dose} = \left(\frac{\text{Weight of Animal}}{F} \right) \cdot g \cdot V \text{ c.c.} \dots\dots\dots(2)$$

where g is the smallest convenient fraction of the volume V which can be injected into the animal accurately (e.g. $V/20$) and

$$F = \frac{U \cdot g}{d} \cdot x \dots\dots\dots(3),$$

and d is the fraction of the full dose it is required to give per unit of animal (e.g. 1, $1/2$, 0.75, etc.).

If, in determining the volume of the dose for a given animal, V is taken as unity and g as fractions of V in divisions of the graduated instrument used for measuring the dose, then if the part in brackets of equation (2) is worked out it gives the number of divisions of the value of g which have to be injected. This part of the calculation can usually be done mentally and by this means it is as easy and as rapid a process to inoculate a number of animals of varying weights as if all were of the same weight.

It follows from equation (3) that the value of F varies inversely as the value of d , and in practice it is found that F determines the maximum and minimum weight of animal to which it is reasonably possible to give a desired dose; therefore, the smaller the value of d the greater the weight of the largest animal which can be used conveniently, when x represents a determined integer.

This method is capable of general and easy application and consequently animals can be dosed in strict proportion to their weight, whatever it may be, without the tiresome necessity of selecting a sufficient number of animals of specified weights for a particular experiment; an ideal seldom realised.

(f) *The influence of sub-culture and age* are extremely important in their capacity for decreasing virulence and the writer knows no medium which will fail to exhibit the well-known attenuating influence on the meningococcus of repeated sub-culture, even when practised at fairly short intervals (12 hourly). Therefore it is very important, when comparing the virulence of cultures of

the meningococcus, only to use growths which have been subjected to the same number of generations on a standard routine medium; the question dealt with in subsection (*d*) seems to require that the second generation from the stock culture should be used.

In the course of this investigation the stock cultures have been maintained on Dorset's egg medium in paraffin sealed tubes, but experiments are in progress with other media. The writer has taken the precaution of keeping a record of every sub-culture made of any strain in the form of a genealogical tree and the growth used in each experiment is carefully correlated with this record; thereby certain features of the ageing of cultures have been noticed, of which the following are examples.

Starting with a culture on egg of known virulence and inoculating mice only with the second generation on strictly comparable media, the observed decrease in virulence with age is shown in Table 1. The transitory recovery of the virulence with sub-culture *on to egg medium* is to be noticed, but the duration of this degree of virulence is less than in the case of the preceding culture and repetition of the process is without effect, perhaps because the influence of repeated sub-culture intervenes.

Table 1.

Egg A	Age of culture in days			M.L.D. for 20 gms. of mouse, in grammes of living meningococcus
	Egg B from egg A at 347 days old after raising virulence	Egg C from egg B when 51 days old	Egg D from egg C when 17 days old	
1	—	—	—	0.0080
16	—	—	—	0.0080
70	—	—	—	0.0120 (= 50 % M.L.D.)
347	—	—	—	0.0160
Sub-cultured 5 generations on egg and 96 on agar	—	—	—	Virulence raised by <i>in vitro</i> method and sub-cultured on to egg B
—	5	—	—	0.0040
—	12	—	—	0.0040
—	26	—	—	0.0040 (= 50 % M.L.D.)
—	27	—	—	0.0080
—	40	—	—	0.0160
—	51	—	—	Sub-cultured on to egg C
—	—	5	—	0.0040
—	—	12	—	0.0160
—	—	17	—	Sub-cultured on to egg D
—	—	—	1	0.0160

M.L.D. = Minimal Lethal Dose.

The accumulation of such observations has enabled the writer to gauge the virulence of a culture or to select a culture of required virulence for some particular experiment by reference to the "genealogical tree," which has justified its existence by economy in the lives of animals and in augmenting the precision of experiments.

(g) *Summary.* In order to establish the minimal lethal dose of living meningococcus culture, that is, to ensure that it will not vary from day to day in an arbitrary manner, it is important to observe:

- (1) That a sufficiently large proportion of the cocci used are viable.
- (2) That the experimental animals are in good health.
- (3) That the nutrient medium affords a growth of approximately constant properties (particularly percentage of moisture).
- (4) That the phase of growth is on the up grade.
- (5) That the dose bears a strict relation to the body weight of the experimental animal.
- (6) That the culture is not unduly aged nor has been repeatedly sub-cultured since the last time of testing its virulence.

III. THE GRADUATION OF DOSES AND THE SELECTION OF THE MINIMAL LETHAL DOSE.

The graduation of a suitable series of doses of living bacteria and the selection of that amount which can be designated one minimal lethal dose involves problems which are, at the same time, of paramount importance and unsolved. Consequently the suggestions presently to be made are to be considered as opinions, based upon experience, which serve very well as a guide until they can be replaced by a rule deduced from proved facts and capable of general application.

The whole question involves the complicated balance maintained by influences contributed by both parasite and host and therefore it is insufficient to base a system of doses upon properties of the parasite alone. It may be considered that the various influences involved resolve themselves into two complex factors which may be conveniently expressed as the "capacity factor" and the "intensity factor."

For want of more precise methods both of these factors are considered by the writer in terms of mass of parasite; a procedure which is inadequate, particularly so in the case of the capacity factor. The capacity factor is regarded as the mass of parasite the actual presence of which is incompatible with the survival of the host and its precise measurement has not been achieved; nor is it possible at the present time to anticipate its full significance. Notwithstanding this difficulty, it is possible to estimate whether the capacity factor diverges widely from, or approximates to the measured dose received by a given animal, and it is this "index of adaptation" which expresses the degree to which a parasite is adapted to invade a given experimental animal. Where the capacity factor approximates to the measured initial dose, as in the case of the meningococcus, the graduated doses used to titrate the minimal lethal dose of a culture, need to vary by relatively large amounts in order to obtain clear cut results; for it must be remembered that under these circumstances the actual amounts injected must be large as it is almost a question of overwhelming the animal by the dose itself. This condition contrasts

strongly with the case of parasites better adapted to invade laboratory animals, where the capacity factor diverges widely from the initial measured dose (e.g. pneumococcus), where the reverse is true because the animal host is overwhelmed by the ready multiplication of organisms introduced in small numbers. It appears that the capacity factor is a variable dependent on the systems of forces contributed by both parasite and host which determine virulence, and there is, at present, no indication of the possible range of variation of this factor. But it seems to the writer that another factor is needed to account for the apparently irregular variation of the estimated index of adaptation and such that it may be considered to determine the magnitude of the initial measured dose required to allow of the capacity factor functioning. This second factor, the intensity factor, is associated with the fact that the significant difference between effective doses diminishes as the quantity constituting a minimal lethal dose decreases. This requires that the difference between the small doses in a series be much less than the difference between the large doses. Therefore a series in arithmetical progression is theoretically unsuitable and one in geometrical progression satisfies the requirements.

For the present the conditions for the meningococcus are amply satisfied by a series in geometrical progression, because of the large doses required, the magnitude of the amount constituting a significant difference, and the variations introduced by the imperfectly adapted circumstances of cultivation of that organism *in vitro*.

The value of the common ratio of a series is determined by both factors: the index of adaptation, which depends upon the capacity factor, is a guide which suggests whether the successive doses are to vary by large or small amounts, and the intensity factor, gauged by the magnitude which constitutes a significant difference in the region of one minimal lethal dose, suggests how large the common ratio need be. The amount which constitutes a significant difference is best estimated by comparing the doses which are just sufficient to kill certainly with those which will kill some though not all of the animals so dosed.

It may be expected, from what has been said in this and the preceding section, that the meningococcus requires a large initial dose which approximates to the capacity factor, therefore the common ratio for a series of doses of that organism in geometrical progression will need to be of a low value. In the writer's opinion 0.5 minimal lethal dose of meningococcus is to be considered small in comparison with 1.0 minimal lethal dose, because such a dose kills comparatively few animals; that is to say, that $(1/2)$ is reckoned small in comparison with unity; $(1/2)^2$ is a magnitude of the second order, etc. Therefore, for a series of doses of living meningococcus in geometrical progression the common ratio adopted is 2, when mice are used. For the sake of comparison, it may be stated that 10 has been found to be a suitable common ratio for a similar series of doses of pneumococcus for mice.

The selection of that amount which constitutes one minimal lethal dose

is always a matter of difficulty, because of the variation in resistance of individual animals and the margin on either side of any selected dose within which a slight variation might give a different result to that obtained.

So far as the animal is concerned, the essential condition which must be imposed is that the dose, when the total mass is given at one time by a stated route, shall be sufficient to kill all the animals so dosed; it must be what Pasteur called a 100 per cent. fatal dose. From this it follows that owing to individual variation in resistance only certain of the animals will receive exactly one minimal lethal dose and the rest rather more than sufficient to kill. The other difficulty is not so easily overcome because no very definite limiting requirement can be set to control it. The starting point of a series of doses is determined by such factors as measurable quantities, volumes which are safely tolerated by the animal of choice, the number of available animals, etc., but more particularly by the judgment of the investigator born of experience. It is important only to maintain a technique which enables the various experiments to be faithfully comparable amongst themselves and readily repeated by other observers.

Thus it is that what is designated "one minimal lethal dose" of a parasite, cannot be considered to represent the point on a curve where the defence of the host reaches zero, but, rather, a selected field in an area representing that phase of a system in which all the hosts die.

IV. THE VARIATION IN VIRULENCE OF FRESHLY ISOLATED STRAINS.

During the course of these investigations opportunity for examining freshly isolated strains has been insufficient to allow of definite conclusions.

The writer is indebted to Dr M. H. Gordon, C.B.E., C.M.G.; Dr A. Stanley Griffith, Major A. S. G. Bell, O.B.E.; and Dr W. H. Scott for the strains he has received. Almost all the cultures available are very old, in some cases dating to 1915, and repeated sub-culture has deprived them completely of whatever virulence they may have possessed.

The scarcity of material only allows of the statement that the freshly isolated strains varied considerably in virulence, as measured by experiments with mice, and that this property exhibited some degree of independence of the endotoxin value of the strain. Gordon's experiments (1920, p. 23) show instances of similar conditions and it is hoped that it will be possible to investigate these points when the arrangements for the collection of freshly isolated strains, which are being made on the part of the Medical Research Council, have been completed.

The need for such an investigation is emphasised by Gordon's (1920, p. 22) observation "That the pathogenicity of the coccus alive and dead, *i.e.* between the virulence and toxicity, was far less in the case of the older cocci than in some, though not in all, of the most recently isolated ones."

V. THE FATE OF MENINGOCOCCI INJECTED INTRAPERITONEALLY INTO MICE.

The establishment of the conditions required for the determination of a minimal lethal dose for the living meningococcus, of itself a matter of interest to immunologists, is of importance because it allows of the investigation of questions relating to the virulence of that organism and the failure to define this primary unit has resulted in the impossibility of titrating the anti-infective power of anti-meningococcal serum.

The limitations of our knowledge of the virulence of the meningococcus is summarised by Dopter (1921, pp. 425-426) and emphasised by the failure experienced by such experimenters as Dopter, Kolle, Wasserman, Gordon, Amoss and others in all attempts to raise the virulence of that organism.

In order to appreciate the conditions determining the raising and lowering of the virulence of the meningococcus to be examined in this paper, it is essential to study the fate of the cocci injected intraperitoneally into mice and rats.

During the early stages of the investigations it was desired to select the more virulent of the available stock cultures for use as antigens in immunising horses. For this purpose mice were used as the test animal and the most favourable route was found to be intraperitoneal. However, it proved very difficult to kill mice with any degree of certainty by injecting living meningococci of strains which had been kept so long in culture.

When an occasional mouse, perhaps less resistant than its fellows, succumbed, the meningococcus was sought for in its body fluid by microscopical and cultural methods, usually without success. But as very large doses of cocci had been introduced into the peritoneal cavity it was inconceivable that all trace of these should be completely obliterated during the time the mouse survived, not more than 14 to 20 hours. In the post mortem examination of such mice, besides the general signs of a purulent peritonitis, chiefly indicated by the character of the scanty peritoneal exudate in which meningococci could seldom be found, the most striking feature of the abdominal cavity was the tightly rolled up omentum. It was here, embedded in the omentum and within its folds, together with masses of phagocytes, that the meningococci were found in large numbers and the picture presented by smears of this organ were suggestive of the fight in progress.

Durham (1897) first observed this collecting action of the omentum, in connection with staphylococci, and Roussiel (1911) has shown that the concentration of phagocytes is always much greater in the omentum than in the peritoneal cavity.

But the uncertain effect of these ancient strains complicated the investigation and it was not until freshly isolated strains were available that the fate of the meningococcus, when injected intraperitoneally, could be examined more extensively, for then it became possible to kill experimental animals more frequently and to compare the effects of variation in virulence.

A mouse dying as the result of multiple intraperitoneal minimal lethal doses of a virulent strain, may exhibit in the peritoneal exudate very large numbers of healthy-looking, well-stained meningococci, of which comparatively few are found to be intracellular. That the presence of such large numbers of cocci in the peritoneal fluid partly depends upon the animal having received an overwhelming dose, is suggested by the examination of other mice, which have died in the course of the same experiment when it is found that the number of cocci seen noticeably decrease with the fall in the dose. It is usual, when working with doses of the magnitude of only one or two minimal lethal doses of a strain of moderately low virulence, to experience difficulty in discovering many meningococci in the peritoneal exudate and quite frequently none can be found. When present they are usually both intra- and extracellular, and it cannot always be said in which situation they predominate. The influence of increased virulence is to augment the number of cocci to be found in the peritoneal exudate and to leave no doubt that the majority are extracellular; but the above-mentioned relation to mass of dose is still apparent. It is under these circumstances that cultures can be obtained from the peritoneal exudate and from the heart blood.

Turning now to the omentum, it is a striking fact that when living meningococci have been injected intraperitoneally, it is only on very rare occasions that cocci cannot be found in this situation; even when they have not been discovered in the peritoneal exudate they have been abundantly present in the omental smear. In the omental smear of a mouse dead as the result of an intraperitoneal dose of living organisms, the extracellular meningococci are in the majority and it is often surprising what massive numbers of cocci are to be seen.

A feature of importance to be considered here is the cytology of the inflammatory exudate. Very large numbers of cells are to be found in the folds of the omentum, but in the free peritoneal fluid they are usually less numerous and they may be scarce when the cocci have been swept up cleanly or when the animal's reaction is poor. The type of cell found under such circumstances as the present, and the time and sequence of their appearance have been thoroughly described by Bordet (1920, p. 173), Dopter (1921, pp. 85 and 99) and others and need not occupy us here, where the character of chief interest is the state of health of the cell.

When the total mass of meningococci, intra- and extracellular, in a given situation is small, the cells for the most part stain normally, but when very large numbers of cocci are present very few healthy looking cells of any type are to be seen; the majority, then, are in various stages of degeneration and fragmentation of the nuclei is a common feature. But this statement needs qualification, for although mass of cocci seems to be the preponderating factor, the observed phagolysis is really proportional to what might be expressed as the "mass of assailing factors."

Thus, remembering the relative distribution of the cocci in the peritoneal

fluid and the omentum, the picture which each situation presents can be realised in all its variations. In animals which have succumbed to a moderate dose, it is common to find that the peritoneal fluid is scanty, almost clear, the few cells seen are healthy, and that cocci are absent or difficult to find; while, on the other hand, the rolled up omentum encloses the exact opposite state of affairs, abundant cocci and very marked phagolysis.

With regard to the ingested cocci and those whose freedom has been regained by the rupture of a cell, various degrees of granular degeneration, typical of intracellular bacteriolysis by leucocytes, are indicated by their stained appearance; and it is a matter of more than passing interest that the amount of degeneration, exhibited by phagocytosed cocci, appears to be to some extent in inverse proportion to the number of cocci present in the cell. Although many such altered cocci are to be found free in the inflammatory exudate bearing no obvious relation to a cell, it is not always possible to say whether their condition is due to intracellular lysis and subsequent liberation, or whether leukines liberated by disintegrated cells or bacteriolysins and alexine have brought about the change; autolysis is not a probable factor. An opinion can sometimes be based upon the grouping of the cocci and the amount of evident phagolysis but even when the influence of leucocytes is strongly suggested there is still ample scope for the action of bacteriolysins in the ordinary sense.

Such are the variations in the microscopical appearance of the inflammatory exudates of mice which are the victims of living meningococci and it is important to compare them with the conditions found in mice killed by injecting killed cocci and with mice that have successfully resisted a dose of living cocci. Thus, though derived from a highly virulent culture, when cocci killed by heating at 55° C., or by desiccation over sulphuric acid, neither of which operations seems to interfere with the endotoxin of the meningococcus, have been dosed in sufficient quantity to cause the death of the animal, it is very rarely that any trace of them can be found in the peritoneal fluid and when present they are almost invariably intracellular. Under these circumstances, too, the omentum is tightly rolled and it is there again that the cocci are found, but it is a striking feature that the majority are intracellular though there is marked phagolysis and liberation of partly lysed cocci. It has been the writer's experience in inoculating a large number of animals with meningococci and their products, that if a mouse or rat looks anything other than extremely ill about 24 hours after inoculation it is unlikely to die, and, if at that period it is sufficiently well to take the least interest in food or its personal comfort it will certainly recover.

Now, 24 hours after inoculation with living virulent cocci, if mice or rats, then so well as to feed and clean themselves, are killed and examined immediately, a condition is found which very vividly contrasts with what has been discussed above. There is an abundant turbid peritoneal exudate containing very large numbers of healthy cells and numerous macrophages busily

ingesting such damaged cells, with their contents, as may be present. The vast majority of cocci are intracellular and whether or not there are *any* free cocci, and also the degree of damage to cells, depends again upon the mass of the dose. Further, and more striking still, the omentum, only rolled at its margin if at all, is frequently stretched between the coils of intestine in all directions, as if when the fight is going well for his side the important stratagem is to increase the area of conflict and provide a large surface for the diapedesis of leucocytes and so to launch these to the attack actually where they are needed. An omental smear presents the same picture as the peritoneal fluid only the concentration is greater.

Three facts of immediate interest must be stated here, though they will receive full discussion in another paper where they properly belong.

(1) The endotoxin of meningococci is liberated, apparently unaltered and in its full strength, both by the leukines extracted from polymorphonuclear cells and by the sensitising substances and alexine of normal serums.

(2) Freshly isolated strains of meningococci have been examined which possess a high degree of virulence and a low endotoxin content. Living doses of the former kill readily and the latter with difficulty, while doses of whole dead cocci of either strain kill with great difficulty, in accordance with what has already been described.

(3) The minimal lethal dose of dead cocci required to kill 20 gms. of mouse represents very many times the minimal lethal dose of extracted endotoxin of the same strain.

It must occur from time to time, in experiments of the nature required by this type of investigation, that, though the animals receiving the larger doses of a series die regularly one or more of those receiving the smallest doses die, although similarly or even more highly dosed animals do not at any time show signs of inconvenience. Irregular results of this kind may be interpreted in the light of what has been said above. On examination the peritoneal fluid and omental smear may show that a good fight has been attempted and considerable phagocytosis accomplished, with the result that relatively few healthy looking free meningococci are found. Nevertheless, for some reason, there is extensive phagolysis and many partially lysed meningococci are seen in the region of degenerated cells. In such a case the interpretation may be allowed that the endotoxin liberated by the leukines has turned the scale. In other cases phagocytosis has been less complete and free cocci are abundantly present; this state of affairs may be labelled "susceptible animal" or "lowered resistance" without advancing matters and frequently a closer approach to the truth is difficult to attain, though sometimes a relatively trifling intercurrent disease may be accepted as explaining the difficulty. An experimental reproduction of this condition, faulty phagocytosis, will be dealt with in the next section.

This state of affairs must not be confused with that which may be described as the survival of animals receiving "multiple minimal lethal doses," a diffi-

culty very seldom experienced by the writer when using *living virulent cocci*, then only in an individual animal and so probably due to some error introduced accidentally. Very irregular results may obtain when working with cultures of low virulence, which may be due either to predominance of non-resisting cocci, or as will be discussed elsewhere, may be due to the lack of some quality in the medium.

From the point of view of virulence the prominent features of the foregoing considerations may be summarised as follows:

(1) The preponderance of free meningococci in the inflammatory exudate is proportional to the virulence of the strain.

(2) When entire meningococci are ingested and *retained* by the phagocytes they cease to take any further part in the conflict and undergo degenerative changes.

(3) In the face of failure of phagocytosis, the power of the leukines liberated through phagolysis is relied upon to destroy the invading organisms, but this is not always successful; with virulent strains many cocci will be found which take the stain deeply and such are often grouped in small clusters as if multiplying rapidly.

(4) When the influence of the meningococcus is such that extensive phagolysis with consequent bacteriolysis is brought about, the endotoxin thus liberated favours the meningococcus in the conflict.

(5) When victory is in the balance the omentum strives to confine the area of conflict and diminish the surface for absorption of toxic products.

But when the experimental animal is definitively master of the situation, the omentum is frequently found spread out and the field of operations is thus extended to the full.

VI. INTERFERENCE WITH THE ACTIVITY OF THE LEUCOCYTES AND THE PERITONEAL FOLDS.

The evidence cited in the preceding section suggests that, whatever the physiological forces at work may be, the virulence of the meningococcus can be expressed in terms of resistance to phagocytosis, and such a view would agree with statements which have been made in respect of other organisms (Bordet, 1920, p. 209; Levaditi, 1914, pp. 484-486). The present section will be devoted to the discussion of further experimental evidence bearing upon the above inference, but it must be recognised at the outset, that, were resistance to phagocytosis the only term of reference used to express virulence, an incomplete statement would result, even though phagocytosis may be the principal factor in natural immunity.

Although the mechanism immediately responsible for the death of the animal properly belongs to another investigation, it is of more than passing interest that individual animals are found to exhibit less than the average degree of resistance to meningococcal infection. Such a state represents an *apparent increase* in virulence of the culture used and the problem is thereby

posed as to the possibility of reproducing the condition in the experimental animals of average powers of resistance.

Besredka's important experiments (1899) demonstrate that the amount constituting a fatal intraperitoneal dose of As_2S_3 varies inversely as the number of leucocytes present in the peritoneal cavity, and, further, that animals which have previously received an intraperitoneal dose of finely powdered carmine succumb to a dose of As_2S_3 which normally they would have tolerated. The effect of the carmine is to encumber the leucocytes.

Roussiel (1911) describes similar experiments, particularly in relation to the function of the omentum and shows that when the pedicle of the spleen has been ligated death of the animal invariably supervenes only when the omentum has been put out of action by excising it, or by a previous dose of carmine, or carbon.

In the literature concerned with the problem of phagocytosis there are numerous instances demonstrating that interference with the activities of the leucocytes favours infection by bacterial parasites but very generally the authors are content to use their experiments as arguments contributing to the controversial discussion of humoral and cellular immunity and the point of view of the parasite is usually neglected.

In the present section, the evidence of experiments interfering with the cellular defence of the host will be considered only in its relation to the raising and lowering of the virulence of the parasite.

An intraperitoneal dose of 0.01 gm. of carmine powder in no way affects the apparent health of mice and the subsequent condition found varies with the time which elapses between dosing and killing the animal for examination. Recoux (1897), Durham (1897), Heger (1904), and others show that carmine is taken up almost completely by the omentum within 15 minutes of intraperitoneal injection. It is first "agglutinated" into masses by mucin as described by Gengou (1908) and then gathered up by the peritoneal folds; the omentum is the principal of these, but the pelvic folds and those in relation to the genital organs are almost equally active. At an early stage (12-20 hours) carmine has no other visible distribution but microscopical examination shows that a fair proportion of leucocytes wandering in the peritoneal fluid contain carmine. The peritoneal folds are tightly rolled and literally crammed with carmine and leucocytes. Four days after injection the carmine is more widely distributed; besides being massed in the peritoneal folds it chokes the lymphatics of the diaphragm and mesenteries, colours the lymphatic glands of the abdomen and thorax and is found in the liver, spleen, and even, contained in leucocytes, in the heart blood and lungs.

The leucocytes free in the peritoneal cavity contain carmine. After three weeks there is no free carmine in the serous cavity but the peritoneal folds are still tightly rolled and crammed with carmine and leucocytes, many of which are overburdened with the pigment. The larger masses of carmine are almost embedded in leucocytes. Carmine is very readily taken up by leucocytes but

to other substances such as finely divided chemically pure carbon and aleurone they appear to behave rather differently.

Numerous experiments with living meningococci, on the lines of those of Besredka with As_2S_3 , show conclusively that in the presence of carmine, given either some hours before or at the same time as an injection of cocci, the apparent virulence of the culture is markedly raised. The following experiment with a strain of moderate virulence will serve as an example.

An 18 hour culture of Type I Netley, second generation from a 27 days old egg culture, was suspended in 0.85 per cent. solution of NaCl in a concentration of 0.0080 gm. living per c.c. The other substances injected into the mice were a 1 per cent. suspension of levigated carmine and 2 per cent. peptone broth, both sterilised in the autoclave for 20 minutes at 120° C. Each dose was duplicated for the result of inoculating single mice is often misleading. The result of these inoculations is shown in Table 2.

Table 2.
Intraperitoneal dose of

	Carmine in gms.		Broth in c.c. 18 hrs. before meningo.	Meningo. gms. living per 20 gms. of mouse	Result
	At same time as meningo.	96 hrs. before meningo.			
A	0	0	0	0.0080	Both died
	0	0	0	0.0040	One died
	0	0	0	0.0020	Both lived
B	0	0.005	0	0.0080	Both died
	0	0.005	0	0.0040	One died*
	0	0.005	0	0.0020	Both died
C	0	0	1.0	0.0080	Both died
	0	0	1.0	0.0040	Both lived
	0	0	1.0	0.0020	One died
D	0	0.005	1.0	0.0080	Both lived
	0	0.005	1.0	0.0040	One died
	0	0.005	1.0	0.0020	One died
E	0.005	0	0	0.0080	Both died
	0.005	0	0	0.0040	Both died
	0.005	0	0	0.0020	Both died
Control	0	0.005	0	0	Killed and examined at the time that the other mice were inocu- lated with meningo.

* The greater part of the dose of meningococcus leaked back after injection in the mouse which survived this dose.

Post mortem examinations. The control mice were killed and examined at the time the other mice were inoculated with meningococcus and showed the typical appearance of mice dosed with carmine four days previous to examination. It is only necessary to emphasise the fact that the carmine found in the peritoneal exudate was all intracellular and that many cells were crammed with pigment.

Series A. These mice revealed the picture that has already been described under such circumstances as the present, within the usual limits of variation. It suffices to emphasise that masses of cocci were present in the tightly rolled omentum and that phagolysis and bacteriolysis were marked.

Series B. Here the distribution of carmine only differed in degree in different situations according to the animal and the general picture of the carmine was identical with that exhibited by the control mice. There were, however, large numbers of cocci in the peritoneal cavity, both extra- and intra-cellular, but many of the latter were liberated owing to phagolysis. Bacteriolysis was quite marked. The omental smear closely resembled that of the control mice and differed only in that a very occasional cell was found containing meningococci and these, together with a few rare cocci found free, might easily have been derived from the exposed surface of the omentum in making the smear. The possibility of migrating cells must not be overlooked in this connection. The striking feature was that phagolysis hardly existed and that cocci, to all intents and purposes, were absent.

Series C. The findings in this series were similar to those of series *A*, except that the majority of the cocci were intracellular and healthy looking cocci were fewer in number. Phagolysis and bacteriolysis were very marked.

Series D. The two mice which died in this series may be considered not to affect the experiment. One exhibited no cellular reaction whatever as judged by the peritoneal exudate and had comparatively few cells even in the omentum. The other was but little better. Both showed gram negative bacilli in the films.

Series E. In this experiment these mice were only examined macroscopically. The carmine was confined to the omentum and genital folds and only very little was found free in certain of the mice. Careful microscopical examination of many mice treated similarly to those of this series has been made, controlled by mice treated as in series *A*, when it has been found that the majority of the large numbers of cocci present in the peritoneal exudate are free and that the cells are chiefly encumbered with carmine. Although phagocytosis and bacteriolysis are evident, phagolysis appears to be less intense in the presence of the carmine. In the omentum cocci, cells and carmine are more abundant but their respective relations are those already described for the peritoneal exudate.

The surviving mice of this experiment were perfectly well when killed, 75 hours after inoculation, and presented the picture of recovered mice; healthy cells were the rule and aged polymorphonuclear leucocytes were being actively ingested by macrophages, and in an occasional one of these the remains of meningococci could be made out.

This and other experiments of the same nature (see Table 4) demonstrate very clearly that the apparent virulence of the meningococcus, when injected intraperitoneally, is greatly increased by interference with the functions of the phagocytes and omentum by means of carmine. At first sight it would appear that the protective influence of the omentum preponderates, but this is not the case, for a more critical examination shows:

(1) That such series as *B* and *E* are virtually identical owing to the phagocytes in the peritoneal exudate of the mice of series *B* being greatly encumbered with carmine.

(2) That although the minimal lethal dose has remained apparently unaltered in spite of the increased number of leucocytes in series *C*, there has been an enormous reduction in the numbers of free cocci compared with series *A*. Further, in other experiments than the one quoted, the "apparent virulence" has at times been reduced by the previous injection of broth, and at other times the mice have been killed very irregularly and so contrast strongly with the controls.

(3) That a very marked reduction in the apparent virulence is evidenced by series *D* in spite of the omentum being completely out of action in this series. These mice must be compared with those of series *B* and *E*, and it must be remembered, when considering this series, that, although the choking of the lymphatics and decreased surface for absorption may have played a part, the mice of series *B* were placed under identical conditions.

Thus it has been proved possible experimentally to increase the *apparent virulence* of the meningococcus by interference with phagocytosis and in this way to reproduce in the average mouse the condition that appeared to be responsible for increased susceptibility in the occasional mouse.

Further, by increasing the numbers of phagocytes present at the time of inoculation of living meningococci, it is possible to reproduce the opposite condition, that of apparent decrease in virulence of the culture.

It must not be supposed, however, that the measurement of successful phagocytosis or resistance to phagocytosis allows of the complete expression of virulence; something has already been said concerning the double effect which bacteriolysis may produce, route and rate of absorption are open to consideration and the importance of the localising influence of the peritoneal folds must also be remembered in the special case of intraperitoneal infection. These are only the more obvious factors involved in the study of the complicated subject of virulence and this determines the position that it is only the resultant of the opposing forces that can be measured and then only in terms of survival or death. Nevertheless, this and the preceding section demonstrate that the apparent virulence of the meningococcus, as determined by intraperitoneal inoculation of mice and rats, depends very largely upon resistance to phagocytosis and the bacteriolytic substances liberated by phagolysis.

VII. THE RAISING OF THE VIRULENCE OF THE MENINGOCOCCUS BY ANIMAL PASSAGE.

Pasteur's magnificent researches and their extension by other observers, have established that the virulence of many parasitic micro-organisms can be decreased at will by the application of appropriate methods. The process has been shown ordinarily to involve either singly or in combination, according to the nature of the parasite, the use of oxygen, light, heat, desiccation, chemical poisons, age or repeated sub-culture. Without entering into details, it will be admitted not to be beyond the bounds of possibility that these influences interfere with, or perhaps even suppress certain unknown important

physiological processes of the micro-organism which are assailing powers and thus the aforesaid balance existing between parasite and host is upset. The complexity of this relation is demonstrated by the particular cases in which the virulence of certain parasitic bacteria is decreased for one species by raising it for another.

It is a matter of every-day experience to the immunologist and often a disconcerting one, that the maintenance of virulence of certain microbes is a matter of difficulty. Under such circumstances the usual practice is to resort to animal passage or constantly to procure freshly isolated strains.

In order that the method of animal passage may be applied it is essential to establish a minimal lethal dose and Dopter (1921, p. 85) summarises the general experience of bacteriologists with regard to the meningococcus as follows: "... , but nothing is so variable, on the whole, as the dose capable of causing death. One sometimes believes oneself to have grasped what one agrees to call the fatal dose of a particular strain, when a new experiment shows that animals, placed under identical conditions, resist, without one understanding why, a dose ten times as great as that previously established."

Such, too, was the writer's experience at the commencement of this investigation, but, with the gradual establishment of the conditions dealt with in Section II, this difficulty gave place to the possibility of being able to ascertain with certainty the minimal lethal dose of a given living culture, which remained constant so long as it was reasonably possible to exclude the influence of attenuating factors and the virulence was not accidentally raised. These conditions can be maintained for a week and even longer, as is shown in Table 1, but the ideal nutritive medium has not yet been found and consequently the influences of age and necessary sub-culture cannot be indefinitely excluded. Even were it possible constantly to obtain freshly isolated strains, there are, for certain purposes, decided advantages in knowing the properties of a selected strain, nor is every culture from human disease of standard virulence.

So it became important to investigate the conditions required for raising the virulence of the meningococcus. That such conditions exist for that organism, when subjected to animal passage, has been shown by Bruckner and Christeanu (1906) although Dopter and R. Koch (1909) and others admit complete failure.

In attempting to raise the killing power of the meningococcus by animal passage, considerable difficulty was experienced in recovering the micro-organism from mice which had succumbed to a dose of a culture of "low virulence"; nor was this difficulty much modified by resorting to cultures of "higher virulence," in which cases passage frequently succeeded at first but very soon the series was broken by failure to recover the meningococcus; and this obtained whether the method used was alternating cultivation and passage or direct transference of heart blood or peritoneal exudate from mouse to mouse. One factor contributing to this failure is the difficulty of maintaining an adequate dose of viable cocci, as indicated by the growth obtained from

the body fluids of dead mice. But what amounts to a mere difficulty in technique of dosage is not sufficient to explain why the killing power of a culture, recovered from a mouse or rat, is raised on one occasion and lowered on another, without exhibiting any definite sequence; nor can it account for the great variation in the number of viable organisms discoverable in animals even when a strain of high killing power has been used.

On the evidence described in foregoing sections it was assumed, as a working hypothesis, that the individual cocci composing a culture might vary considerably among themselves in the degree of their adaptation to parasitic existence and that the phagocytes and leukines might exercise a "selective" action. Under such circumstances, if the cocci of considerable parasitic capacity only survived, the maximum virulence of a strain would be obtained in a single passage; but should the resistance of the host be low, it would then be possible for the cocci of mean virulence for the particular culture to grow and as these are, presumably, in the majority the virulence of the strain recovered from the animal would be unchanged or even lowered by a single passage.

This hypothesis has been borne out by experiment. It has been possible to show that cultures recovered from the peritoneal cavity of animals (mice or rats), all of which received a fatal dose of a given culture at the same time, in certain cases had been raised in virulence as much as fourfold (incidentally as high a level as that strain has been known to reach), in other cases the virulence had remained stationary and in yet others it had been lowered.

Moreover, when mice have received a dose of carmine and meningococcus at the same time and the resistance of the host is thus hampered, in the manner already described, the apparent increase in virulence usually results in a more ready recovery of the strain from the animal, but, so far, no evidence has been forthcoming that the *actual* virulence of the strain has been raised. On the contrary, strains which have been recovered from mice which have been overwhelmed by the meningococcus, either by administering multiple minimal lethal doses, or by interference with the defensive mechanism of the animal, have usually shown a decrease in *actual* virulence.

Thus it is possible to raise the virulence of the meningococcus by animal passage, but the process is by no means straightforward and certain, on account of the uncontrollable variations in the defensive mechanism of the experimental animal. In point of fact, animal passage, as applied to the meningococcus, is essentially a matter of chance and consequently unsuitable for routine application in the production of therapeutic serums. The lack of suitable experimental animals is probably responsible for this condition.

VIII. THE RAISING OF THE VIRULENCE OF MENINGOCOCCUS *IN VITRO*.

The accumulated evidence points significantly to the "selective" action of the phagocytes and liberated leukines being the determining factor in the raising of virulence of the meningococcus. Consideration of the fate of the

meningococcus inoculated intraperitoneally and the examination of strains recovered from animals so inoculated, demonstrate the uncontrollable variability of the factors contributing to what can be generally expressed as the resistance of individual experimental animals.

These various factors at present defy complete analysis; but, in the case of experimental intraperitoneal meningococcal infection with purpose to raise the virulence of a strain, it is desired to realise:

(1) The conditions affording optimal physiological activity of the leucocytes and leukines.

(2) The conditions which enable the meningococcus to exercise its pathogenic properties only by the manifestation of its most complete physiological adaptation to a parasitic existence.

Occasionally these ideal conditions may be realised in a given experimental animal, but though it is possible to intensify the assailing powers of the meningococcus by animal passage, it is still beyond our powers to reproduce at will exactly the conditions required.

The conception that individual cocci composing a culture vary in their degree of adaptation to a parasitic existence suggested some experiments *in vitro* in which the leucocyte retains its role of "selective" agent. But because it is very difficult to maintain leucocytes under conditions ensuring their optimal physiological activity, the use of living leucocytes introduces as much uncontrollable variation as does the use of the whole living animal. Considering now, that when prevailing conditions cause very extensive phagolysis the range of action of any lysed cell is extended and that the concentration of leukines thus liberated may be such that it is immaterial whether the cocci are ingested or not, for they are then practically subjected to the conditions imposed by phagocytosis, extracts of leucocytes therefore were substituted for the living cells in these experiments.

Leukines are substances of unknown composition around which a vast literature has grown, which has been lucidly analysed by Levaditi (1914) and reveals the complexity of the experimental conditions it is desirable to realise.

The mode of extraction finally adopted by the writer was that described by Gengou (1921), because the substance obtained in this manner appears to be, almost certainly, that which produces bacteriolysis within the leucocytes themselves and its properties do not allow of its identification with either alexine or a proteolytic ferment. The cells used are the polymorphonuclear leucocytes of the rabbit and their extracts, made by Gengou's method, are remarkably bacteriolytic, as has been claimed for them by Gengou, and relatively small quantities will destroy vast masses of meningococci. That their action is non-specific and that immunisation does not produce a quantitative augmentation, the writer is able to confirm. The method used to collect the leucocytes appears to affect the potency of the extract obtained; this the writer measures in terms of the time required and the number of polymorphonuclears represented in the amount of extract which is sufficient to cause the

death of 0.0010 gm. of living meningococci, as determined by culture and not by the microscopical appearance of the cocci, using proper controls. It is surprising to notice the degree of change in the appearance of the cocci which is yet compatible with a relatively abundant growth. The most potent extracts have been obtained when the leucocytes have been collected by intrapleural injection of 20 c.c. of isotonic broth. A much greater yield is obtained by injecting a hypertonic solution containing 1 per cent. peptone and 1 per cent. to 2 per cent. of NaCl (see Bordet 1920, p. 183; Le Play and May, 1911), but in this case the extracts have been less powerful; the addition of potassium and calcium salts to the hypertonic solution produced no appreciable difference.

When hypertonic solutions are used the amount of fluid recovered, in 12 to 16 hours, from the pleural cavity of the rabbit exceeds the amount injected and when the cells have been removed, a transparent, yellow fluid remains which, as the writer's experiments show, possesses the properties of the Gengou extract of the washed cells and can be used in its stead.

When isotonic solutions are injected very little fluid remains after 16 hours and its properties have not been investigated, but the large amount of fluid remaining after seven hours has similar properties to that obtained with hypertonic solutions. It is found that the potency of extracts is not adequately expressed by the number of leucocytes represented by each c.c. of extract and it is desirable that further investigation be undertaken to determine the factors influencing this variation.

The independent variables controlling the reaction appear to be time, temperature, and relative concentration of the reacting substances, but the rule for their adjustment to obtain a desired result has not yet been determined and the present procedure is one of trial.

As both the culture and Gengou extract selected for a given experiment will vary respectively in degree of virulence and potency, compared with similar selections used in other experiments, the range of reaction in respect of time and relative concentration, when the temperature is kept constant (37° C. being the optimum), must be determined for any desired combination.

Although the Gengou extract alters but slowly when kept on ice, the fact that the properties of the culture change rapidly with age and sub-culture, ordains that only the shortest possible interval should intervene between the titration of the range of reaction and the attempt to recover a virulent strain from a given culture by the selective action of a Gengou extract; usually both stages of the experiment can be performed at one time.

The method at present employed is to select relative concentrations of the chosen culture and Gengou extract, such that the total mass of meningococci present is not destroyed within a time less than 48 hours, at 37° C.; the cultures obtained towards the end of that time have been found invariably to be more virulent than the original culture, or the control culture subjected to identical treatment only excepting the influence of Gengou extract.

The following experiment is given to show the method employed:

The weighed growth from a 20 hour culture, second generation from an egg culture, was emulsified in broth in a concentration of 0.0400 gm. living cocci per c.c.

An equal amount of this emulsion was placed in each of five tubes, to each of which was added the desired amount of Gengou extract, broth and 0.85 per cent. NaCl solution, such that each contained an identical concentration of broth, excepting tube 2 which only contained 60 per cent. of the desired amount because of the volume of extract it was desired to add.

The relative concentrations of the reacting substances together with the results of culture are shown in Table 3.

It must be stated that the leucocyte extract used in this experiment is the most powerful the writer has made and it has only been equalled by two others.

Table 3.
Titration of Potency of Gengou Extract.

Tube No.	Living meningo. in gms. per c.c. of mixture	Amount of Gengou extract in c.c. per 0.0010 gm. meningo.	Growth on sub-culture after contact at 37° C. for the time indicated in hours							
			0	6	23	27	48	72	120	144
1	0.0080	0	+++	++	+++	+	+++	+++	+++	+++
2	0.0080	0.08 (= 72×10^4 polymorph)	+++	+	1 col.	0				
3	0.0080	0.04 (= 36×10^4 polymorph)	+++	++	++	+	0			
4	0.0080	0.02 (= 18×10^4 polymorph)	+++	++	++	+	++	+	0	0
5	0.0080	0.01 (= 9×10^4 polymorph)	+++	++	+++	++	++	+	+	0

+++ = Very good growth.

++ = Good growth.

+ = Poor growth.

0 = No growth.

The microscopic changes were followed closely at the same intervals of time as the viability, but it is not thought necessary to burden the paper with these long descriptions, although they are a helpful guide and of some interest.

At this stage an 18 hour culture, the second generation from the same egg culture which provided the growth for the above titration, was divided into aliquot parts: (1) the first part was inoculated into mice, (2) the second was subjected to the action of the above Gengou extract, in a concentration of 0.03 c.c. (= approximately 27×10^4 polymorphs) per 0.0010 gm. of living meningo., (3) and the third portion was placed under identical conditions as (2) but substituting 0.85 per cent. NaCl solution for the Gengou extract. The second and third mixtures were then placed at 37° C.

This concentration of extract was chosen as it was estimated that the total mass of cocci would not be destroyed in less than 80 hours, thus allowing time for the multiplication of such cocci as would survive the antagonism of the extract.

Under these conditions the culture obtained after 75 hours' contact was selected for animal inoculation with the results given in Table 4.

Table 4.
(Each dose was given to two mice.)
Intraperitoneal dose of

Toxin	Series	Living meningo. in gms. per 20 gms. of mouse		Carmine in gms. at same time as coccus	Result
Culture untreated with Gengou extract	1	0.0160		0	Both died
		0.0080		0	One died
		0.0040		0	One died
	2	0.0080		0.005	Both died
		0.0040		0.005	One died
Culture after 75 hours' treatment with Gengou extract	3	0.0040		0	Both died
		0.0020		0	Both died
		0.0010		0	One died
	4	0.0040		0.005	Both died
		0.0020		0.005	Both died
		0.0010		0.005	Both died

Considering only the mice which did not receive carmine (series 1 and 3), it is evident that the minimal lethal dose for the strain of meningococcus used has been raised from 16 mgms. to 2 mgms.; that is to say that before treatment with Gengou extract the amount of this strain which constituted one minimal lethal dose was 1/1250 part of the body weight of the mouse and after the virulence was raised, in the manner described, the same result was obtained by a dose equivalent to 1/10,000 of the body weight of the animal. This represents a very considerable increase in virulence for the meningococcus. In other experiments the time required has been shortened by varying the concentrations.

The writer has never yet failed to raise the virulence of a culture by this method, but the degree to which it has been raised varies because the exact conditions required have not been determined. So far as present experimental evidence goes it seems to indicate that increased concentration of potent extract, with conjugate shortening of the viable period, imposes such conditions that a given culture of low virulence does not attain to as high a degree of killing power as it does when the conditions are less exacting. One point of importance respecting this statement must be emphasised: namely, that in order to obtain a marked increase in virulence the extract used must be strongly bacteriolytic; it is expedient to employ a low concentration of a strong extract rather than a high concentration of a weak one.

It is incorrect to suppose that it is sufficient merely to place meningococci in contact with an extract of leucocytes, and, at some time before the complete extermination of the cocci, to recover a culture which will be raised in virulence, as if it were simply a matter of destruction of the weaklings and survival of individual cocci of full assailing power pre-existing in the original culture. That this is not the state of affairs has been indicated, time and again, by the fact that cultures recovered from contact with a high concentration of potent

extract, have failed to reach the degree of virulence attained by the same strain in the same experiment subjected to the influence of a very much lower concentration of the same leucocyte extract. In fact in certain experiments the cultures recovered from contact with a high concentration of extract have actually shown a diminution in killing power.

This point is illustrated by the following experiment chosen as a typical example from among several others.

A 20 hours' growth, second generation from an egg culture, was emulsified in 0.85 per cent. NaCl solution in a concentration of 0.0200 gm. of living cocci per c.c. Part of this emulsion was inoculated into mice and the intraperitoneal minimal lethal dose proved to be 0.0080 gm. of cocci for 20 gms. of mouse. The remainder was equally distributed between three tubes each containing 0.25 c.c. of broth for each milligramme of cocci added. The final volume was adjusted so that the concentration of cocci was 0.0010 gm. per c.c., by adding physiological saline alone to tube *A*, and Gengou extract and physiological saline to the other two, so that tube *B* contained 0.5 c.c. of extract (equivalent to 51.7×10^5 polymorphs) and tube *C* 0.1 c.c. of extract to each milligramme of cocci. These three tubes were then incubated at 37° C. for 42 hours and the sub-cultures grown from each of them at that stage were emulsified in 0.85 per cent. NaCl solution and injected intraperitoneally into mice. The minimal lethal dose of these cultures for 20 gms. of mouse proved to be 0.0080 gm. of cocci for tube *A*, 0.0040 gm. for tube *B*, and 0.0020 gm. for tube *C*.

The culture used in this experiment was one of low virulence and the extract was rather on the weak side; the concentrations used were determined by previous experiments.

It appears to the writer that the reaction is partly controlled by a delicate balance of antagonistic factors resulting in an adaptation on the part of the living cocci of an evolutionary nature. When it is required to raise the virulence of a strain, the writer sets up a series of varying concentrations and follows their progress by cultural and microscopical methods, and periodical animal experiment.

No evidence has been obtained to indicate that the leucocytes of immunised animals yield an extract exhibiting a greater or less degree of efficiency than those of normal animals. A point of general interest is raised by the fact that extract of rabbit leucocytes are capable of raising the virulence of meningococci for mice and rats.

It must be mentioned here, that, although fresh normal guinea-pig serum exhibits an extremely marked bactericidal action on the meningococcus, the writer has not succeeded in raising the virulence of that organism by means of such serum; although it is also true that the virulence of the cultures used was not lowered by the use of such serum.

Specific bacteriolytic sera have not, so far, been used by the writer. When hypertonic solutions have been injected into rabbits, the fluid recovered, freed of cells by centrifuging, has given identical results to those obtained with

leucocyte extracts of moderate potency; this property survives even after the fluid has been heated at 55° C. for 30 minutes.

One more observation must be mentioned as it might confuse the issue if not recognised. It is frequently found that the control tube, containing no Gengou extract, gives cultures which show a slight increase in virulence compared with the original culture. The degree of virulence attained is very seldom comparable to that reached by cultures from tubes in which the correct concentration of extract obtains, nor is it constant or progressive, but fluctuates. In some ways it resembles the influence of sub-culture, previously discussed, and it is probably closely connected with certain factors influencing virulence which will be discussed in another paper.

The convenience of this *in vitro* method of raising the virulence of the meningococcus, the degree of control that can be exercised over the conditions of experiment, and the rapidity and certainty with which the desired result is obtained, constitutes, in the writer's opinion, a decided advance on the method of animal passage. Many points remain which require experimental investigation and their interest and practical importance is undeniable.

The writer has refrained from discussing the time factor in detail because its influence is still under investigation. But that does not detract from its importance and it is desired to emphasise it, by stating that when cocci are placed in contact with a suitable concentration of leucocyte extract to yield cultures of enhanced killing power, the virulence is raised progressively with time; but the time factor appears to have a limiting value, for with excessive action a slight reversion of virulence has been observed. The properties of leucocyte extracts and details of the methods for their preparation will not be discussed as the references to the literature amply cover the subject, to which but little has been added by the writer.

IX. THE INFLUENCE OF DESICCATION ON VIRULENCE.

It has long been recognised that drying in air, at room temperature or at 37° C., on cotton wool, filter paper, or other porous substance is detrimental to the life of the meningococcus (Dopter, 1921, p. 81; Vines 1916, p. 13; Treadgold, 1916, p. 14) and it is claimed that under these conditions, whether in the form of cultures or contained in mucus, the meningococcus dies in 6 to 12 hours. Reference has already been made to the fact that desiccation over H_2SO_4 *in vacuo* is rapidly fatal to the meningococcus and this emphasises the interest in the method described by Swift (1921) for preserving cultures of various organisms, including the meningococcus, depending upon drying the frozen growth over P_2O_5 and glycerine at very low pressures. (It has long been shown that the meningococcus survives temperatures as low as -20° C., Dopfer, 1921.)

It must be emphasised that these apparent contradictions are evidence that the observations are incomplete, so that the general law determining the influence of desiccation on the meningococcus has not been formulated.

Drying on swabs and materials exposed to the air at variable temperatures involve many uncontrolled factors and for experimental purposes the method has, therefore, a limited application. When attention is directed to desiccation over H_2SO_4 *in vacuo* at 37°C ., one factor, which is not generally recognised, must be taken into account: that is the vapour pressure of H_2SO_4 . This matter first attracted the writer's attention, when working with dysentery bacilli during 1916-19, owing to the relatively copious deposits of sulphates on the walls and platform of the desiccator and the sides of vessels containing the large masses of growth under investigation from which ammonia or amines were constantly liberated. The writer is inclined to blame this appreciable partial pressure of H_2SO_4 for the death of the meningococcus under such conditions, because that organism is readily recovered from growth which has been dried, until its weight becomes constant, over NaOH or P_2O_5 at 40 mm. Hg pressure and at 37°C . These are special cases, but they serve to show that the meningococcus readily survives desiccation, without recourse to the elaborate technique of Swift, and their application to further study of the question of virulence is of interest.

When a given culture of meningococcus is killed by desiccation over H_2SO_4 , the minimal lethal dose bears the same relation to that of the living culture as does the minimal lethal dose of cocci killed by heat; in both cases a very much larger dose of the dead cocci is required to kill; as much as twenty times the dose of living cocci may be necessary. This statement holds good even for those strains of which the minimal lethal dose of extracted endotoxin represents a smaller mass of cocci than does the minimal lethal dose of living cocci.

When, however, the cocci have been dried to constant weight over NaOH or P_2O_5 , *in vacuo* at 37°C . or by Swift's method, the cultures recovered from the desiccate have not altered in virulence. Furthermore, when the desiccate itself is emulsified and inoculated into animals it kills in exactly the same dose as did an aliquot part of the growth used for desiccation, and cultures of meningococci can be recovered from the mice.

Thus it appears that the assailing factors possessed by a virulent strain of meningococcus are not interfered with by even so drastic a measure as desiccation, by which between 80 and 84 per cent. by weight of the original growth has been removed in the form of moisture and gases, so long as the viability of the cocci has not been destroyed.

A more extended generalisation than this cannot at present be justified, because only strains of moderate virulence have been tested and sufficient time is required to discover the influence of age upon the desiccate.

X. DISCUSSION.

(a) *On the minimal lethal dose.*

It is claimed, in this paper, that it is reasonably possible to establish a minimal lethal dose for a given culture of meningococcus, which is constant

over a sufficient period to enable the experimental investigation of virulence to be undertaken. There are defects, only too easily discerned, in the method, but reference to the literature will allow of the concession that a small step has been taken in the right direction. The most obstinate impediment, though, perhaps, not the most obvious but certainly the most important, are the involved questions of the physical conditions, the nutrition and the removal of waste products of cultures, which are essential conditions for the maintenance of the complete physiological activity of the parasite. Questions relating to this part of the problem, the relation of virulence to medium have been omitted purposely from this paper, with the intention of dealing with them separately.

(b) *On virulence.*

Gordon (1920, p. 22-25) demonstrated that killing by ether, heat and desiccation destroyed the pathogenicity of the meningococcus although (p. 43) none of these agents destroy its contained endotoxin. His desiccation experiments are open to the objection that he etherised his material before drying it, even though, in the method he used, the ether boils off *in vacuo* at 37° C., and is absorbed by the H_2SO_4 . Nevertheless it is true that desiccation at 37° C. *in vacuo* over H_2SO_4 does destroy the pathogenicity of the meningococcus when used as *entire cocci*; even in those particular strains which, weight for weight of the same culture, kill 20 gms. of mouse in a much smaller dose when completely broken up than they do when living. This property, thus easily lost by the meningococcus, Gordon called "the labile factor" and he expressed disbelief that it simply depends upon the vital capacity of the coccus, as expressed by growth *in vitro*. Gordon sought without success to discover its origin in a soluble toxin, a haemolytic substance and a ferment, but he showed very clearly that the killing power decreased in proportion to the diminution in number of viable cocci in a given culture. The evidence now brought forward in this paper amply confirms Gordon's far seeing deduction, although no closer approximation is possible than to state that the property is a function of the viability of the organism, partially expressed by its power to resist the mechanism involved in the cellular defence of the host.

Tulloch (1920, pp. 79-81) was unable to obtain evidence, by *in vitro* experiments, that thermolabile antiphagocytic substances are developed by the meningococcus in its growth; but he did observe that the susceptibility of the meningococcus to phagocytosis varied in different cultures. Further, he was unable to obtain cultures which would give consistent results over periods longer than three or four days; but he does not state whether liability to phagocytosis increased with sub-culture. It is noteworthy that he observed no difference between living cocci and those heated at 60° C. for 30 minutes, particularly as this does not conform with observed phagocytosis *in vivo*. It would appear that either or both of two difficult conditions were uncontrolled, namely the "virulence" of the organism and the optimal conditions for the leucocytes to function.

The apparent virulence of the meningococcus can be increased or diminished by appropriate interference with the defensive mechanism of the host. For this purpose substances which are inert in respect of the vitality and integrity of the leucocytes and bacteria must be used, such as isotonic broth and washed carmine, and not an inoffensive microbe such as *B. subtilis* (*B. prodigiosus* was used by Dopter (1921, p. 86)), because the writer has observed that *B. subtilis* is capable of liberating the endotoxin of the meningococcus and dysentery bacillus apparently unaltered and also is capable of living in the peritoneal cavity of mice and even producing fatal results. Dopter also failed to bring about a like result by the use of leucotoxic serums; perhaps this was due to the liberation of leukines (see Bordet, 1920, p. 348; Yoshinaga, quoted by Levaditi, 1914, p. 489).

It appears that the evidence of the literature, when taken in conjunction with that produced in this paper, suggests strongly that the capacity of the meningococcus to invade the living tissues rests upon an undefined property, depending upon the life of the coccus, which can be expressed by resistance to phagocytosis. But additional evidence has been produced that this alone is not sufficient to express completely the degree of virulence of the culture, which is not simply a property peculiar to the parasite, but depends upon a balance of a variety of factors contributed by both host and parasite.

The degree of virulence is measured by the intensity of the factors contributed by the parasite which is just sufficient to overcome the intensity of the factors contributed by the host and can be expressed in terms of the mass of parasite required to bring about the death of the host. But this minimal lethal dose is an unsatisfactory "unit" because of its liability to variation with small derangements of the contributing factors, whether originating in host or parasite, which cannot at present be estimated.

For the present this measurement of virulence must suffice, and, with the recognition of its shortcomings, it can be made to serve a useful purpose in the study of variation in virulence due to alteration in factors contributed by the parasite.

(c) *On the raising of the virulence of the meningococcus.*

Very few authors claim to have raised the virulence of the meningococcus; Bruckner and Christeanu (1906) did so by intraperitoneal passage in rabbits and Ruppel claims to do it by a process which he refused to divulge (see Dopter, 1921, p. 425), but Dopter and other acknowledged authorities did not succeed in their various attempts. Up to the present no very precise analysis has been made of the conditions which it is essential to observe, and, in view of the general admission of failure in respect of the meningococcus, the writer feels that the contribution set forth in this paper is a step towards bridging this gap in our knowledge.

The fact that in the presence of a high concentration of Gengou extract a given culture does not yield so virulent a strain as it does when subjected to

a lower concentration, with conjugate lengthening of the viable period, suggests that the raising of virulence is not merely a selection of pre-existing resistant individual cocci, but, rather, an appropriate balance of factors, which affords the optimal conditions for the survival and multiplication only of such organisms as are capable of developing physiological properties tending to endow them with a more complete adaptation to parasitic existence.

The convenience, rapidity and unfailing success of the *in vitro* method described, are its greatest recommendations, although it is not without theoretical interest and perhaps it may prove capable of general application by appropriate use of various types of cells.

It is a matter of interest that Alexander (1918), working with pneumococci, found that both sensitised cocci and cocci incubated at 37° C. for 6–8 hours with leucocytes were too virulent to use in large intravenous doses to immunise rabbits; but, that sensitised cocci incubated with leucocytes were attenuated though they did not lose their vitality.

My sincere thanks are due to the Medical Research Council for affording me the unhampered opportunity of attacking the subject in my own way, under conditions conducive to scientific achievement.

I wish, too, to record my grateful appreciation of the unfailing and unselfish assistance afforded me by Mr R. Ayrton throughout these investigations. Without his skilled and enthusiastic co-operation it would not have been possible to have covered so much ground in this and other investigations.

XI. CONCLUSIONS.

1. It is possible to establish an intraperitoneal minimal lethal dose for living meningococcus of any strain, which remains constant over a period determined by the rate of loss of "virulence" of the culture consequent on its growth *in vitro*.

2. The minimal lethal dose of a culture so determined and expressed in terms of the mass of living cocci which is just sufficient to kill "unit" weight of experimental animal of average resistance for the species, is an incomplete expression of virulence; but it is a sufficiently close approximation to render it possible to appreciate certain factors contributing to virulence and to study their individual variation and influence.

3. Virulence is not a property of the parasite alone, but is the resultant of physiological forces exerted by both parasite and host. Variation in any factor contributed by either participant materially affects the experimental measurement of this resultant, so that an *apparent* increase or decrease of virulence is readily effected.

4. Resistance by the meningococcus to the mechanism and processes of phagocytosis, is the expression of undefined properties peculiar to the parasite contributing to virulence. This resisting power of the parasite depends upon it

being alive and becomes more evident as the amount constituting the minimal lethal dose decreases in mass.

5. It is possible to increase or decrease the virulence of a strain by animal passage, but the extent and direction of the change cannot be predicted in any experiment, because the determining factors are at present beyond complete analysis and control in the living experimental animal.

6. The virulence of a given culture of meningococcus can be raised with certainty *in vitro* by subjecting it to the influence of a suitable extract of polymorphonuclear leucocytes. The amount of change produced depends upon temperature, time and the relative concentration of the active agents.

An optimum exists for each of these variables: that for temperature (37° C.) is dependent upon the very nature of the leucocyte extract, whilst those for time and relative concentration are dependent upon one another and are determined by the nature of the reaction and the activity of the reacting agents.

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DURATION OF PASSIVE IMMUNITY

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PART IV.

(With Charts XVII—XXI.)

PART II of this paper dealt with the rate of disappearance of antitoxic horse serum from normal rabbits; this present section deals with the rate of disappearance of antitoxic horse serum after injection into rabbits previously sensitised to horse serum. A few cases of serum sensitised rabbits have already been reported; Part I (Table III) recorded the results of injecting antitoxic horse serum into seven rabbits sensitised from 3 to 11 months previously with one or more injections of 1/100th c.c. or more of horse serum. The conclusions drawn were that the course of elimination of antitoxin followed the same three phases that occur when antitoxic horse serum is eliminated from normal rabbits, but that Phase C occurred earlier and was more rapid. In Part II (Table VII) we showed that similar rates of loss occurred in three rabbits sensitised six or seven weeks earlier with 0.5 c.c. of horse serum, and Table VI recorded the results of experiments on two rabbits which received their second injection 10 and 11 days after the first; in these two rabbits the usual phases were to a certain extent masked by residual precipitin formed in excess in response to the previous injection. A number of results in Part II also suggested that apparently normal rabbits had become naturally sensitised to horse serum; it was of interest therefore to determine the sensitising effect of various amounts of horse serum. As a preliminary experiment four rabbits were injected subcutaneously with 1/100,000th c.c., 1/1000th c.c., 1/10th c.c.

and 10 c.c. of horse serum, and four weeks later injected intravenously with antitoxic horse serum; the curves of subsequent antitoxic content were then plotted. The results obtained are recorded in Tables XXXV and XXXVI and Curves 65–68 on Chart XVII. The exceptional Phase A loss shown by rabbit 65 is probably due to an error in titrating the sample of blood taken 15 minutes after injection; calculation from the weight of the rabbit would give the antitoxic value as 8.1 units and would thus reduce the Phase A loss to 53.7 per cent. The four rabbits exhibit a progressive rate of elimination or increasing degree of sensitisation in accordance with the volume of the sensitising dose. Rabbit 66 was definitely sensitised by a subcutaneous injection of 1/1000th c.c. of horse serum but rabbit 65 that had received 1/100th of this amount did not give so conclusive a result. Table XXXVII has been compiled to show the number of days taken for the

TABLE XXXV.

Showing the antitoxic value of four rabbits, previously sensitised with different quantities of horse serum, at different intervals of time after the intravenous injection of 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Rabbit	65	66	67	68
Weight	1840	1840	1530	1590
Sensitising dose in c.c.	1/100,000	1/1000	1/10	10
Interval	4 weeks	4 weeks	4 weeks	4 weeks

Time interval	Antitoxic value in units per c.c.			
15 minutes	11.0*	7.5	9.5	9.5
1 day	3.75	4.0	4.5	4.5
2 days	3.0	3.25	2.75	3.0
3 "	2.25	2.25	1.62	1.0
4 "	2.0	1.8	0.005	0.015
5 "	1.62	0.22	<0.0005	<0.0005
6 "	0.55	<0.0005	—	—
7 "	0.02	—	—	—
8 "	70.0005	—	—	—

* This reading is probably incorrect; the antitoxic value calculated from the weight of the rabbit is 8.1 units per c.c.

TABLE XXXVI.

Showing the percentage daily loss in antitoxic value of the blood of four rabbits previously sensitised with different quantities of horse serum, at different intervals of time after the intravenous injection of 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Time interval	Rabbit 65		Rabbit 66		Rabbit 67		Rabbit 68	
0-1 day	65.9*	Phase A	46.7	Phase A	52.6	Phase A	52.6	Phase A
1-2 days	20.0	} Phase B Av. 18.8	18.7	} Phase B Av. 23.1	38.9	} Phase B Av. 39.5	33.3	} Phase B Av. 82.6
2-3 "	25.0		30.7		40.1		66.7	
3-4 "	11.1		20.0		99.7		98.5	
4-5 "	19.0	} Phase C Av. 81.1	87.7	} Phase C	—	} —	—	} —
5-6 "	66.0		—		—		—	
6-7 "	96.3		—		—		—	

* The observed value for the antitoxic content on the first day was probably too high (see footnote to previous table); the calculated value gives a Phase A loss of 53.7 %.

TABLE XXXVII.

Showing the number of days taken for the antitoxic content of the rabbits recorded in Tables XXXV and XXXVI to fall below various stated fractions of the total amount injected.

Rabbit	Serum injected	Weight	1/10	1/100	1/1000	1/10,000
65	Horse	1840	6	7	8	8
66	"	1840	5	6	6	6
67	"	1530	4	4	4	5
68	"	1590	4	4	5	5

antitoxic content of these rabbits to fall below 1/10th, 1/100th, 1/1000th and 1/10,000th of the amount injected. A comparison between Table XXXVII and Table XIX in Part II shows that rabbit 65 eliminated antitoxin more quickly than the majority of normal rabbits. Omitting rabbits over 2½ kilos, out of 19 rabbits recorded, in only one (the exceptional case of rabbit 94) had the antitoxic content fallen to 1/100th or 1/1000th earlier than in rabbit 65 and only one other rabbit reached either level in such short time. The 1/10,000th level was reached by rabbit 65 in less time than that taken by any other rabbit of the same weight or less. The degree of responsiveness of this rabbit resembled that of rabbits weighing 2½ kilos or more. The high rate of percentage loss during Phase B in rabbits 67 and 68 in Table XXXV, indicated also by the steepness of that section of the curves on Chart XVII, may be taken as showing that excess

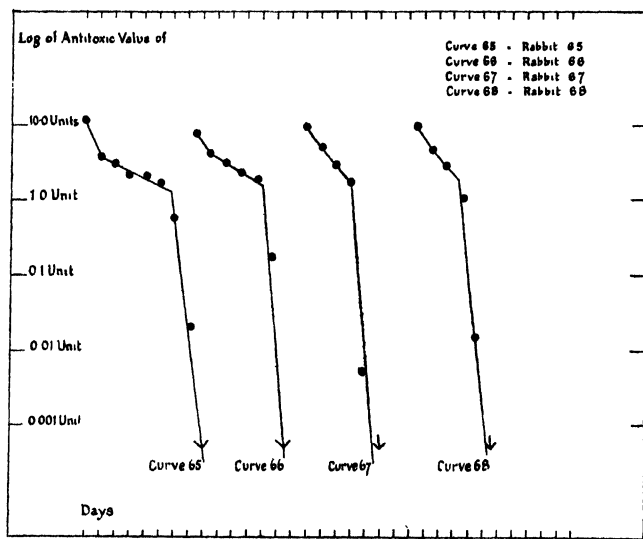


Chart XVII

precipitin was present four weeks after a first injection of 1/10th c.c. and a first injection of 10 c.c. of horse serum respectively. It would appear from this experiment that so small a dose as 0.00001 c.c. of horse serum causes an appreciable degree of active immunity to horse serum, but more experiments would be needed definitely to confirm this statement.

The next experiments were designed to show the progressive sensitisation that occurs after a series of injections of horse serum into rabbits. Table XXXVIII shows the course of elimination of antitoxic horse serum after each of four intravenous injections in two different sensitised rabbits, 13 and 33. The first injections were given nine and six months respectively after the last small sensitising dose; the curves of resultant antitoxic content after the first injection have already been published in Part I (Curves 4 and 7 on Chart II). On re-injection five weeks later, rabbit 13 lost 74.1 per cent. of antitoxin

within the first 24 hours and all antitoxin injected was lost in three or four days. It must be pointed out here that the base line in the charts relating to the sensitised rabbits recorded is not always zero because there may be some degree of active immunity to diphtheria present. (The rabbits used had received injections of toxin antitoxin mixtures and were thus actively immune to diphtheria toxin and sensitive to horse serum.) Thus the antitoxic value of rabbit 13 before the first injection here recorded was 0.005 unit per c.c. as the result of active immunity produced by some earlier stimulus; later the degree of residual active immunity had fallen to 0.003 unit per c.c. Six weeks after the second injection of 0.5 c.c. of antitoxic horse serum there was sufficient residual precipitin present immediately to eliminate 50 per cent. of the horse serum then injected. Fifteen minutes after the injection the antitoxic value of the blood was less than 50 per cent. of that calculated according to the weight of the rabbit, and 24 hours later only 0.1 per cent. remained. The fourth

TABLE XXXVIII.

Showing the antitoxic value of two sensitised rabbits, after a series of four intravenous injections of 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Rabbit ...	13				33			
Interval since previous injection	9 mths.	5 wks.	6 wks.	6 days	6 mths.	3 wks.	10 wks.	6 days
Time interval	Antitoxic value in units per c.c.							
15 minutes	5.0	6.75	2.5	3.0	8.0	8.0	9.5	0.02
1 day	—	1.75	0.006	0.003	4.5	1.1	2.75	0.003
2 days	2.5	1.1	—0.003	—	3.5	0.45	1.6	0.0015
3 "	1.6	—	—	—	2.25	0.02	0.0015	—
4 "	0.005	0.005	—	—	0.03	—	—	—
5 "	—	—	—	—	—	—	—	—
6 "	—	—	—	—	—	—	—	—
7 "	—	—	—	—	—	—	—	—

injection was given six days after the last and again rapid elimination occurred and no passive immunity remained 24 hours after injection.

Very similar results were obtained with rabbit 33. After the second injection, 86.2 per cent. of the antitoxin was lost in the first day and all passive immunity had disappeared by the third day. Rapid loss also occurred after the third injection given ten weeks after the second. Six days later the horse serum injected was eliminated at so great a pace that only 1/400th of that injected could be detected within 15 minutes of the injection.

A similar experiment recorded in Tables XXXIX and XL, was carried out on two other rabbits, 38 and 54, but the subsequent injections of horse serum were given subcutaneously. Rabbit 38 had already received an intravenous injection of 0.5 c.c. of antitoxic horse serum as recorded in Table III, Part I. Three weeks later the same amount was injected subcutaneously and the highest antitoxic concentration in the blood, reached two days later, was 0.05 unit per c.c., 1/50th of the value that a normal rabbit would exhibit

at the same interval of time after a similar injection. Six days later ten times as much antitoxic horse serum was injected and the highest value reached, again on the second day, was 1/10th that of the previous occasion, or 1/5000th of the value a normal rabbit would have possessed. The effect of the injection apparently was to use up much of the residual precipitin without acting strongly as a secondary stimulus, because the next subcutaneous injection given four weeks later resulted in a much higher titre. The maximum value recorded, on the third day, was little less than 1/10th

TABLE XXXIX.

Showing the antitoxic value of a sensitised rabbit after a series of subcutaneous injections of unconcentrated horse serum containing diphtheria antitoxin.

Rabbit 38

Previous history Two sensitising doses subcutaneously of 0.013 c.c. followed three months later by 0.5 c.c. intravenously

Interval since previous injection	3 weeks	6 days	4 weeks	4 weeks	5 days
No. of units injected	750	7500	7500	7500	7500
	Antitoxic value in units per c.c.				
Time interval					
1 day	0.004	0.00015	0.04	0.003	0.04
2 days	0.05	0.005	1.75	0.003	0.022
3 "	0.001	0.005	2.0	0.0012	0.0015
4 "	—	0.0018	0.002	—	0.0005
5 "	—	0.0012	—	0.0005	—
6 "	—	0.0008	0.001	—	—
7 "	—	?	—	—	—

TABLE XL.

Showing the antitoxic value of a sensitised rabbit after a series of subcutaneous injections of unconcentrated horse serum containing diphtheria antitoxin.

Rabbit 54

Previous history One sensitising dose intravenously of 0.025 c.c.

Interval since previous injection	5 weeks	7 weeks	5 days
No. of units injected	750	7500	7500

	Antitoxic value in units per c.c.		
Time interval			
1 day	2.5	18.0	2.25
2 days	3.75	30.0	3.0
3 "	2.75	25.0	2.75
4 "	1.9	—	2.0
5 "	0.005	2.75	Killed
6 "	<0.0005	Reinjected	—

of the titre expected from a normal rabbit. Subsequent injections, four weeks and again five days later, resulted in very low titres. This rapid elimination of antitoxin is of great importance clinically if man becomes sensitive to horse serum as readily as the rabbit. It is pointed out that rabbit 38, after two small subcutaneous injections of horse serum followed three months later by an intravenous injection of 0.5 c.c. horse serum and again three weeks later by a further injection of 0.5 c.c. subcutaneously, was so sensitive six days after the last injection that the highest concentration in the blood, after a sub-

cutaneous injection of 5 c.c. of horse serum, represented only 1/15,000th of the total antitoxin injected. A later injection after an interval of four weeks from the previous one, showed no higher value. The results following the third subcutaneous injection of this rabbit are of particular interest, and would appear to indicate that all residual precipitin was used up in eliminating the majority of the antitoxin absorbed into the circulation during the first day, for during the next two days absorption was normal, and then further production of precipitin was stimulated.

Rabbit 54 was in an earlier stage of immunity to horse serum; the course of elimination of antitoxin after the first two injections recorded may be considered as typical for subcutaneous injections into sensitised rabbits. The antitoxic content after the next injection of 7500 units given five days after the previous injection, was so reduced by excess precipitin that the values recorded corresponded very closely with those following the first injection when only 750 units were given.

The next case to be considered concerns a rabbit, number 61, injected intravenously with 0.5 c.c. of antitoxic horse serum at intervals of approximately a week. Tables XLI and XLII record the antitoxic content and rate of loss after each of a series of nine injections. The first eight injections were of the same amount, but at the last injection one-tenth of the usual quantity was given. The speed of elimination was sufficiently rapid after the last two injections for the 15 minute reading to be below the theoretical value. After the first three injections over 90 per cent. of antitoxin present immediately after injection was lost during the subsequent 24 hours. Curves 69 to 77 on Chart XVIII show graphically the rapidity of elimination after each injection. Each curve suggests a large initial loss which uses up most of the excess precipitin from the previous injection, and is then followed by a Phase B loss accelerated by continued precipitin formation. Curves 70 and 71 and possibly 73 exhibit all three phases, but the other injections appear all to have been lost before Phase C commenced. It is significant that in no instance was all antitoxin eliminated within the first 24 hours. In most instances 98 per cent. of the 750 units injected was lost within 24 hours; when only 75 units were injected the first day loss was again 98 per cent. of the amount injected.

Rabbit 48 recorded in Table XLIII was injected intravenously every day with 0.5 c.c. of antitoxic horse serum for 16 days, and then at irregular intervals during the next week. On the twenty-fourth day of the experiment the animal received its twentieth and last injection of antitoxic serum, but the same volume of normal horse serum was administered on six occasions during the next seven days. Many factors are involved in this experiment, and the issues are not very clear, but a number of interesting points may be gathered from a detailed consideration of Table XLIII and Chart XIX. The first injection is followed by the usual 50 per cent. loss due to Phase A; if the curve of elimination of the antitoxin given at each injection follows an independent course, then, for each dose of antitoxin in turn, Phase A will be succeeded

by Phase B, which will continue until Phase C, induced by the first injection, interferes with the total antitoxic content. Thus, assuming Phase A to be approximately 50 per cent. and Phase B to be approximately 25 per cent., then for every 100 units injected the second day content would be 50 before injection and 150 after injection. Before the third injection there would remain 37 units from the first injection and 50 from the second. The following

TABLE XLI.

Showing the antitoxic value of a rabbit after a series of intravenous injections, given at short intervals of time, of unconcentrated horse serum containing diphtheria antitoxin.

Rabbit 61									
Injection no.	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Interval in days since previous injection	—	11	7	8	6	7	4	6	6
No. of units injected	750	750	750	750	750	750	750	750	75
Antitoxic value in units per c.c.									
Time interval	10.5	—	8.0	10.0	9.5	8.5	11.0	6.5	0.11
15 minutes	4.0	3.25	1.8	0.16	0.015	0.16	0.08	0.25	0.0015
1 day	3.0	2.0	1.1	0.015	0.006	0.04	0.015	0.05	0.0005
2 days	—	0.80	0.015	0.0015	0.004	0.004	0.002	0.0045	—
3 "	1.5	0.03	—	c. 0.0015	0.0015	0.0015	0.0015	—	—
4 "	0.8	0.0015	—	—	? 0.0005	—	0.001	—	—
5 "	0.14	—	—	? 0.0005	—	—	0.0005	—	—
6 "	0.02	? 0.0005	—	—	—	—	—	—	—
7 "	0.001	—	< 0.0005	—	—	—	—	—	—
8 "	< 0.0005	—	—	—	—	—	—	—	—
9 "	—	—	—	—	—	—	—	—	—

TABLE XLII.

Showing the percentage daily loss in antitoxic value of a rabbit after a series of intravenous injections given at short intervals of time, of unconcentrated horse serum containing diphtheria antitoxin.

Injection no.	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Time interval									
0-1 day	61.9	—	77.5	98.4	99.8	98.1	99.3	96.2	98.6
1-2 days	25.0	38.5	38.9	90.6	60.0	75.0	81.2	80.0	66.6
2-3 "	—	60.0	98.6	90.0	33.3	90.0	86.7	91.0	—
3-4 "	29.3	96.2	—	—	62.5	62.5	25.0	—	—
4-5 "	46.7	95.0	—	—	—	—	33.3	—	—
5-6 "	82.5	—	—	—	—	—	—	—	—
6-7 "	85.7	—	—	—	—	—	—	—	—
7-8 "	95.0	—	—	—	—	—	—	—	—
8-9 "	—	—	—	—	—	—	—	—	—

day the figure would become 28 for the first, 37 for the second and 50 for the third. Thus the apparent loss for each day would be from 100 to 50, *i.e.* 50 per cent., from 150 to 87, *i.e.* 42 per cent., from 187 to 115, *i.e.* 39 per cent., gradually diminishing each day. The actual figures seen after the second, third, fourth and fifth injections are 24 per cent., 32 per cent., 26 per cent. and 32 per cent. Such a rate of loss only slightly in excess of Phase B loss suggests that repeated injection has the effect of paralysing or blocking the Phase A loss.

TABLE XLIII.

Showing the antitoxic value of a normal rabbit (number 48) during a series of daily injections of 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Days	Antitoxic value in units per c.c.			Percentage loss 24 hours after each injection
	Before injection	15 minutes after injection	Increase	
1	0.000	10.5	10.5	—
2	5.5	12.5	7.0	47.6
3	9.5	17.0	7.5	24.0
4	11.5	21.0	9.5	32.3
5	15.5	23.0	7.5	26.2
6	15.5	22.0	6.5	32.6
7	11.0	21.0	10.0	50.0
8	8.5	16.0	7.5	59.5
9	5.5	14.0	8.5	65.6
10	5.0	14.5	9.5	64.3
11	6.5	14.0	7.5	55.2
12	4.8	17.0	12.2	65.7
13	2.75	15.5	12.75	83.8
14	3.5	17.0	13.5	77.4
15	4.0	13.0	9.0	76.5
16	4.5	12.0	7.5	65.4
17	—	—	—	—
18	5.5	16.0	10.5	32.3
19	7.5	16.0	8.5	53.1
20	—	—	—	—
21	11.0	18.0	7.0	17.1
22	—	—	—	—
23	—	—	—	—
24	13.5	23.0	9.5	9.2

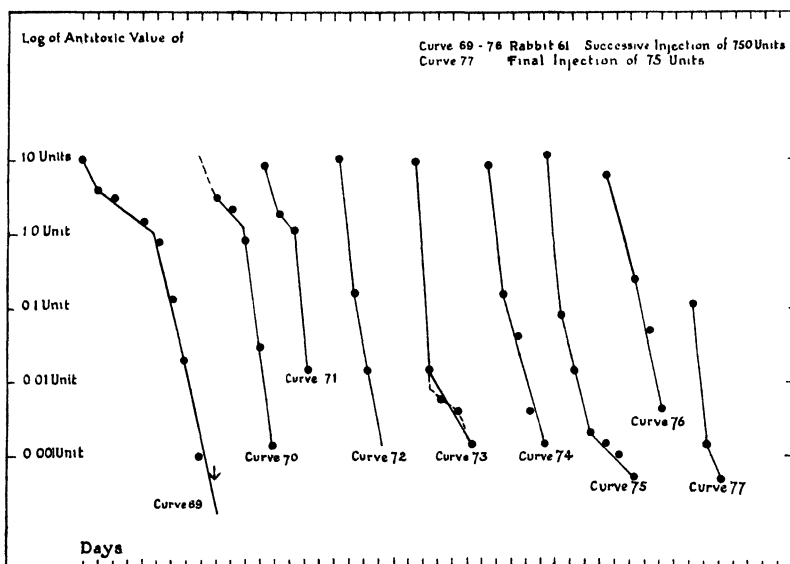


Chart XVIII

By the seventh day precipitin formation has commenced, and the daily rate of loss increases from 50 per cent. on the seventh day to 83.8 per cent. on the thirteenth day. The antitoxic value of the rabbit on the day after 12 successive daily injections of antitoxin is exactly half the antitoxic value possessed on the day after the first injection. After the thirteenth day the rate of daily loss rapidly diminished, and the curve of antitoxic content again rises while precipitin formation lessens, and after the nineteenth day ceases. It would appear therefore that Phase C has also been suppressed or prevented. A possible explanation is that the first few injections only may have acted as stimuli, and the injections given during the subsequent period of active antibody production may have failed as stimuli owing to the rapid destruction

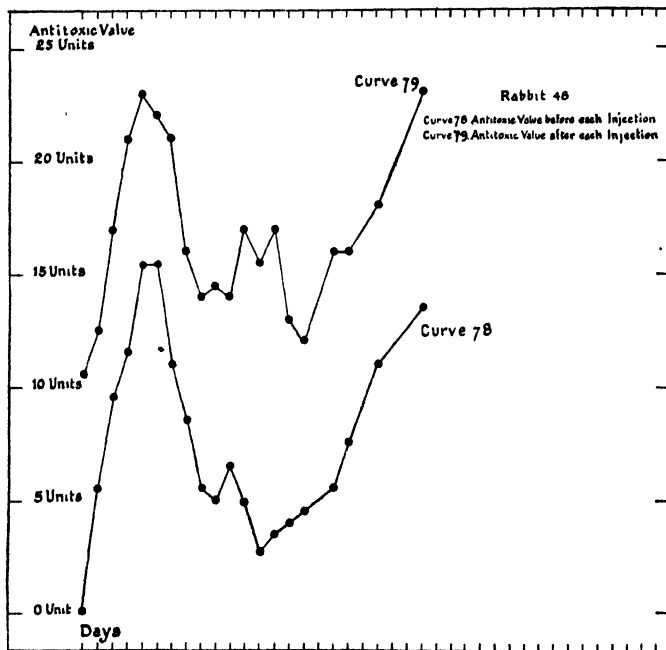


Chart XIX

or elimination of antigen. It is possible, however, that excessive stimulation may have crippled antibody response.

The antitoxic content of rabbit 48 after the last injection of antitoxic horse serum, is traced in Table XLIV and Curve 80 on Chart XX. The daily percentage loss averages 24 per cent. over a period of 40 days, consequently it must be concluded that all loss during the period falls under the heading of Phase B.

Comparing rabbit 48 with the previously recorded rabbit 61, it would appear that a series of injections of antitoxic horse serum at short intervals of time, such as daily injections, may maintain some degree of passive immunity over a long period; at longer intervals of a week each injection is rapidly lost.

In the next experiment recorded in Table XLV and Curve 81 on Chart XX a normal rabbit (number 62) received only one injection of antitoxic horse

TABLE XLIV.

Showing the antitoxic value of rabbit 48 at daily intervals after the last of a series of 20 injections of unconcentrated horse serum containing 750 units diphtheria antitoxin.

Days	Antitoxic value in units per c.c.	Percentage loss since previous day	Days	Antitoxic value in units per c.c.	Percentage loss since previous day
1*	11.5	50.0	21	0.055	8.3
2*	8.5	26.5	22	0.045	18.2
3	—	—	23	0.033	26.7
4*	5.75	17.8	24	—	—
5*	3.75	34.8	25	0.022	18.4
6*	2.75	26.7	26	0.014	36.4
7*	2.25	18.2	27	0.010	28.6
8	—	—	28	—	—
9	1.75	11.8	29	0.0066	20.5
10	—	—	30	—	—
11	1.13	19.7	31	—	—
12	0.70	38.0	32	—	—
13	0.45	35.7	33	—	—
14	0.40	11.1	34	0.002	21.3
15	0.35	12.5	35	—	—
16	0.22	37.1	36	0.0016	10.6
17	—	—	37	—	—
18	0.14	20.2	38	0.0012	13.4
19	—	—	39	—	—
20	0.06	34.5	40	0.0005	35.4

Average 24.3 %.

* Injected with 0.5 c.c. normal horse serum, intravenously.

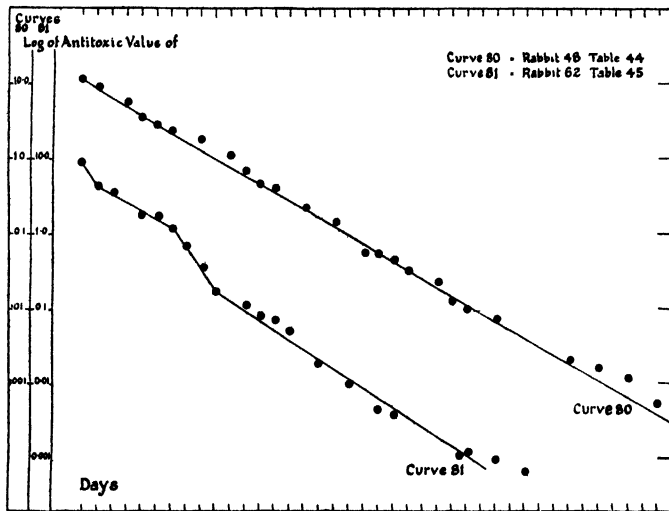


Chart XX

serum followed by daily injections intravenously of 1.0 c.c. of normal horse serum. Phase A is followed by Phase B lasting five days and showing an average daily loss of 21 per cent. Phase C, commencing on the seventh day,

shows an average daily loss of 48·5 per cent.; the small effect is due to the dilution of the antitoxic horse serum by normal serum. After a Phase C, lasting only three days, the percentage daily loss falls to an average of 22·4 per cent. Some degree of Passive Immunity has been maintained for over four weeks by means of daily injections of normal horse serum.

TABLE XLV.

Showing the antitoxic value of rabbit number 62 at different intervals of time after an intravenous injection of 0·5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin followed by daily intravenous injections of 1·0 c.c. of normal horse serum.

Days	Injection	Antitoxic value in units per c.c.	Percentage daily loss	
0	0·5 c.c. antitoxic horse serum	8·5	—	
1	1·0 c.c. normal	4·25	50·5	Phase A
2	"	3·25	23·5	
3	"	—	—	Phase B
4	"	1·9	23·5	Average
5	"	1·62	14·7	21·1
6	"	1·25	22·8	
7	"	0·7	44·0	Phase C
8	"	0·35	50·0	Average
9	"	0·17	51·4	48·5
10		—	—	
11	"	0·12	16·0	
12	"	0·08	33·3	
13	"	0·07	12·5	
14	"	0·05	28·6	
15	"	—	—	
16	"	0·02	36·8	
17	"	—	—	
18	"	0·01	29·3	
19	"	—	—	Phase D
20	"	0·0045	32·9	Average
21	"	0·004	11·1	22·4
22	"	—	—	
23	"	—	—	
24	"	—	—	
25	"	—	—	
26	"	0·0012	21·4	
27	"	—	—	
28	"	0·001	8·7	
29	"	—	—	
30	"	0·0007	16·3	
31	"	—	—	
32	"	0·0007	—	
33	"	—	—	
34	"	70·0005	—	

In a further experiment a normal rabbit, number 60, was injected intravenously at daily intervals for three weeks with 1·0 c.c. of normal horse serum, and then five days after the last injection of normal serum received an intravenous injection of diphtheria antitoxic horse serum. The course of elimination of antitoxin from this rabbit is shown in Table XLVI and Curve 82 on Chart XXI. The contrast between this rabbit and number 48 (Table XLIV and Curve 80) is very marked. Rabbit 48 had received a few more injections than rabbit 60 and showed no signs of accelerated loss; rabbit 60, on the other hand, contained considerable excess precipitin five days after

the last injection of normal horse serum. Four weeks later rabbit 60 was again injected (see Curve 83). The big loss during the first 24 hours shows that

TABLE XLVI.

Showing the antitoxic values of three rabbits at different intervals of time, after intravenous injections of 0.5 c.c. of unconcentrated horse serum containing 750 units of diphtheria antitoxin given after a previous series of injections of horse serum.

Rabbit	60	60	48	62
Previous history	21 injections of 1.0 c.c. normal horse serum at daily intervals	22 injections of horse serum at daily intervals	30 injections as recorded in Table XLIII	34 injections as recorded in Table XLV
Interval since last injection	5 days	4 weeks	7 weeks	11 days
Time interval	Antitoxic value in units per c.c.			
15 minutes	4.75	8.5	10.5	7.5
1 day	0.5	2.25	5.5	1.25
2 days	0.14	1.37	4.25	0.7
3 "	0.003	<0.0005	2.75	0.3
4 "	? 0.0005	—	0.3	0.16
5 "	—	—	? 0.0005	0.055
6 "	—	—	—	—
7 "	—	—	—	0.011
8 "	—	—	—	0.009
9 "	—	—	—	0.004
10 "	—	—	—	0.003
11 "	—	—	—	0.0022
12 "	—	—	—	0.0014
13 "	—	—	—	—
14 "	—	—	—	0.0011
15 "	—	—	—	<0.0005

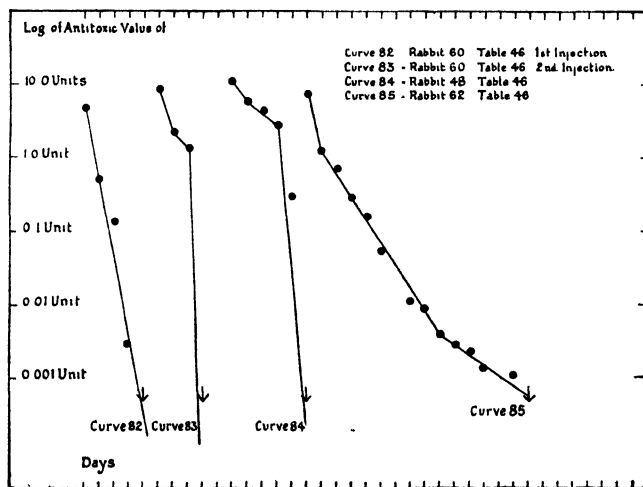


Chart XXI

precipitin was still present, and the general slope of the curve indicates a high degree of active immunity. Tables XLVI and XLVII and Chart XXI also record the results of subsequent injection into rabbits 42 and 62. Rabbit 48,

when reinjected seven weeks after the last injection of the series already recorded, showed no indication that precipitin was present at the time of injection, but the early appearance of Phase C in Table XLVII and Curve 84 shows that the rabbit was actively immune. Rabbit 62, however, when re-injected 11 days after the last injection of the series recorded previously in Table XLV was in a condition of active production of precipitin and for the first nine days Phase A and Phase B losses were increased by precipitin. No accelerated loss occurred because the serum injected in the presence of excess precipitin failed to act as an antigen.

TABLE XLVII.

Showing the percentage daily loss in antitoxic values of three rabbits at different intervals of time, after intravenous injections of 0.5 c.c. of unconcentrated horse serum containing 750 units diphtheria antitoxin given after a previous series of injection of horse serum.

Time interval	Rabbit 60	Rabbit 60	Rabbit 48	Rabbit 62
0- 1 day	89.4	73.5	47.6	83.3
1- 2 days	72.0	39.1	22.7	44.0
2- 3 "	97.8	> 99.0	35.3	57.1
3- 4 "	—	—	89.1	46.7
4- 5 "	—	—	—	65.7
5- 6 "	—	—	—	—
6- 7 "	—	—	—	55.3
7- 8 "	—	—	—	18.2
8- 9 "	—	—	—	55.5
9-10 "	—	—	—	25.0
10-11 "	—	—	—	26.7
11-12 "	—	—	—	35.4
12-13 "	—	—	—	—
13-14 "	—	—	—	11.4
14-15 "	—	—	—	—

At first sight the three rabbits dealt with in Tables XLIII to XLVII do not give consistent results, but for each variation two explanations are suggested: (a) an antigen may fail as a stimulus to antibody production if given intravenously when excess antibody is present, (b) excess antibody produced by former stimuli may be used up by subsequent injections.

SUMMARY OF PART IV.

1. There is some evidence that an injection of 0.00001 c.c. of horse serum causes an appreciable degree of active immunity to horse serum in rabbits.
2. Rabbits actively immune to horse serum receiving a series of injections of antitoxic horse serum at intervals of three to ten weeks eliminated the antitoxin at rapidly increasing rates.
3. An intravenous injection of antitoxic horse serum given six weeks after the second of a series of 0.5 c.c. in a rabbit already actively immune to horse serum was eliminated so rapidly owing to excess precipitin that less than 1/1000th remained 24 hours after injection.
4. Another rabbit which, six days after a previous injection, was given

serum intravenously, lost over 99 per cent. of the antitoxin within 15 minutes of the injection.

5. The maximum of circulating antitoxin detected after the second subcutaneous injection into a sensitised rabbit was only 1/15,000th of the amount injected.

6. Antitoxic horse serum injected intravenously into rabbits at weekly intervals was rapidly eliminated and less than 2 per cent. could be detected 24 hours after each injection.

7. After 12 daily intravenous injections of antitoxic horse serum into a rabbit, the antitoxic content was only one-half of that after the first injection; later injections were not eliminated so quickly and precipitin formation gradually ceased.

8. The rate of elimination of antitoxic horse serum in a rabbit may be greatly delayed by daily injections of normal horse serum.

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A TECHNIQUE FOR MEASURING THE EXCRETION OF BACILLI OF THE ENTERIC GROUP IN THE FAECES OF INFECTED MICE

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A Report to the Medical Research Council.

(With 1 Chart.)

IN recent studies on experimental epidemiology, and especially in a series of reports by Webster (1922, 1923 *a, b, c, d, e, f* and *g*), attempts have been made to standardise certain of the factors concerned in the spread of bacterial infection among mice. One such factor is dosage, to which great importance has been attached by Amoss (1922 *a* and *b*) and also, though to a somewhat less extent, by Webster, whose later work leads him to emphasise the importance of host-susceptibility in determining the form of the epidemic wave. Webster would appear to regard variations in dosage as decisive, in the sense that an effective dose must be applied; but once the critical limit of dosage is exceeded he would give first place to host-susceptibility in determining the subsequent course of events.

The various factors which may play a part in determining the form of the epidemic curve of mortality are considered by one of us in a recently published report (Topley, 1923), and the factor of dosage is discussed at some length. Attention is called to the great difficulty in defining this term, when we turn from experiments in which graded doses of culture are administered by some controllable technique, and consider the spread of infection from host to host. A satisfactory definition will not, however, end our difficulties; we have still to determine in how far the methods at our disposal will enable us to give to the term, as defined, that quantitative expression which is essential if significant results are to be obtained.

It appeared reasonable to assume, in dealing with enteric infection in mice, that the most important path of infection was by the excretion of the causative bacilli in the faeces of an infected animal, and their passage to a fresh host in contaminated food. Acting on this assumption, the problem clearly resolved itself into the practicability of measuring the excretion of *B. enteritidis* Aertrycke (herein referred to as *B. aertrycke*¹) in the faeces of mice, in a way that would allow continuous records to be obtained for each individual mouse, and so enable us to study the distribution of *B. aertrycke* among the population at risk.

¹ The organism referred to is of the "Mutton" variety.

If we consider a mouse-population, among which enteric infection is spreading, we may ask whether the proportion of mice which are excreting the specific organisms at a given moment, or the number of bacilli which are being excreted by any given mouse, or by all the excreting mice together, forms the better measure of the effective dose to which a susceptible member of the population is submitted. A high proportion of excretors will give us a high proportion of the total daily collection of faeces with some degree of infective power, and thus the risk of any given portion of food being contaminated with such material will be high. Massive excretion by a few of the mice will be less effective in ensuring the contamination of a high proportion of the daily food, but may be of decisive importance by increasing the probability that any portion of the food which is contaminated will contain an effective dose of bacilli.

For these reasons, we were prepared, at this stage, to accept as adequate a technique which would serve to detect *B. aertrycke* in specimens of faeces with a considerable degree of certainty, and which would afford an approximate measure of the relative numbers of viable *B. aertrycke* present, when comparing one specimen with another.

THE TECHNIQUE EMPLOYED.

It would serve no useful purpose to outline the various methods and modifications which we tested during the earlier part of this investigation. The technique finally adopted depends upon the power of *B. aertrycke* to produce hydrogen sulphide from peptone, and perhaps from other constituents of the media employed. Lead acetate is added to an agar medium in suitable concentration, and the colonies of *B. aertrycke* are detected by the browning of the colony itself and of the surrounding medium. The medium actually employed differs little from that recommended by Amoss (1922 *a*) for the differentiation of colonies isolated on preliminary plates. The actual method of preparation is, however, a matter of the utmost importance. The addition of the lead acetate solution, during the later stages of preparation, may result in chemical or physical changes which render the medium almost useless as a nutrient material. We do not propose to discuss here the nature of the changes involved, but we would emphasise the importance of rigid adherence to the empirical formula which has sufficed to eliminate these disturbing variations, and which is given in full in the Appendix.

The specimens of faeces are collected by placing each mouse in a specially constructed funnel, with a short wide neck opening into a conical tube, and provided with a loosely fitting cover. The specimens are always collected in the morning, when the cages are changed, and the mice are placed in the funnels *en route* from the dirty cages to the clean. The general method adopted in putting up the cultures for counting follows closely the technique described by Wilson (1922), and we can entirely confirm his views with regard to the advantages of roll-tubes over plates, where large numbers of cultures have to be prepared.

The actual method of preparing the suspensions of faeces, and the subsequent dilutions is as follows:

The faeces are emulsified in a few c.c. of Ringer's solution, 2–10 c.c. according to the amount of faeces present, and the suspensions so prepared are allowed to settle until the coarser particles have subsided to the bottom of the tubes. Each tube of faecal suspension is now placed in a separate rack, and in the same rack are placed (a) a tube of standard diameter containing 10 c.c. of normal saline with 0.5 per cent. formalin, (b) a tube containing 3.96 c.c. of sterile Ringer's solution, and (c) three sterile test tubes, 6 by $\frac{5}{8}$ ins. in size.

For making the dilutions sterile capillary tubes are employed, calibrated to deliver 50 drops per c.c. With one of these about 1 c.c. of the faecal suspension is taken up, 10–40 drops are added to the tube of formalinised saline, the actual number added being noted, two drops are added to the tube containing 3.96 c.c. of Ringer's solution, and one drop is added to the first of the three empty tubes. The tube containing the 1/100 dilution in Ringer's solution is now shaken and, with a fresh pipette, ten drops of this dilution are added to the second empty tube, and one drop to the third. These three tubes are now placed in a rack in a water-bath kept at 40–45° C. and to each is added 2 c.c. of the special medium employed, which has been kept at hand in the melted condition in a second water-bath. The three tubes are immediately rolled under the tap in the usual manner, allowed to set firmly, and incubated for 24 hours at 37° C. in a sloping position with the plugged mouths of the tubes pointing slightly downwards.

The tubes containing the dilutions of the faecal suspension in formalinised saline are set aside until the following day, when the culture-tubes are counted. Counting is carried out with a hand lens, and only the brown colonies are enumerated. The typical *B. aertrycke* colony is sharply defined, deep brown in colour, and with a definite and sharply defined brown halo; but variations from the typical form occur and all brown colonies should be counted, except those which are greyish-yellow rather than brown, and have no halo. Colonies of this latter type are rarely present and do not in practice cause any real difficulty.

Each of the three tubes, containing 0.02, 0.002 and 0.0002 c.c. of the original faecal suspension, is examined. If brown colonies are present in the second or third tubes as well as in the first, only that tube is counted which corresponds to the highest dilution, unless the number of brown colonies in this tube is less than ten, in which case the tube containing the next lower dilution is also counted if the brown colonies are well-formed and distinct. In all cases the highest figure obtained after allowing for the dilution is taken as correct. Sometimes only one tube contains brown colonies, and these are then counted whatever the number may be.

When all tubes have been counted, the dilutions of the faecal suspensions in formalinised saline, which were put up at the same time as the roll cultures, are sorted out; and those are retained which correspond with the culture-

tubes which have given brown colonies, the rest being discarded. These tubes are well shaken, and their turbidity is compared with a graded set of turbidity tubes prepared according to the method of McFarland (1907), but adjusted so that tube 10 corresponds to a suspension of *B. aertrycke* containing approximately 1,000,000,000 bacteria per c.c. When adding the faecal suspension to the tubes of formalinised saline, the number of drops added is adjusted so that the turbidity may fall between 10 and 1 on the scale employed.

From the turbidity reading so obtained, and the number of drops which were added to the 10 c.c. of formalinised saline when the dilution was prepared, we calculate the factor required to reduce the actual count obtained for that specimen to the number of viable *B. aertrycke* per c.c. of a faecal suspension with a turbidity corresponding to 1,000,000,000 bacteria per c.c.; or shortly, and very approximately, we express our results as the number of viable *B. aertrycke* per 1,000,000,000 bacteria in the faeces, assuming the turbidity of the suspensions, after the settlement of the coarser particles, to be due entirely to the bacterial content.

The figure so obtained is based on the count of brown colonies, and it remains to verify the fact that these colonies are colonies of *B. aertrycke*. For this purpose five or ten brown colonies are picked from the positive tube, sub-cultured into small amounts of broth (1-1.5 c.c.) in small test-tubes, and incubated over-night at 22° C. Next morning, an equal amount of saline containing 0.5 per cent. formalin is added to each tube, and the tubes are then heated for one hour at 55° C. Each of these killed suspensions is then tested against high-titre agglutinating sera, agglutination being carried out at 55° C. for two hours. In the great majority of cases all the colonies tested from any one tube will be positive or negative when tested by agglutination. In such cases the figure already entered is retained if the agglutination results are positive, or discarded if they are negative. Where some of the colonies from a given tube are positive and others negative, the figure for the corresponding specimen is corrected accordingly. It is probable that this correction is often unnecessary, and indeed erroneous, the negative results being due to overgrowth of the brown-producing organisms by some other bacterium in the broth sub-cultures; for it is impossible to ensure pure sub-cultures from crowded tubes. On the other hand, such results are sometimes due to the presence of two kinds of brown colonies. It is probably better to correct all figures, where mixed agglutination results are obtained, rather than none; and one rule or the other must, of course, be rigidly adhered to.

To take an actual example:

A specimen of faeces was treated in the manner recorded above. The third tube of the culture series, containing 0.0002 c.c. of the faecal suspension, gave 13 brown colonies.

The corresponding turbidity tube, in which 15 drops (15×0.02 c.c.) of the faecal suspension had been added to 10 c.c. of formalinised saline gave a turbidity of 2, corresponding to 200,000,000 bacteria per c.c.

Thus, the number of bacteria producing brown colonies per c.c. of a suspension of this specimen of faeces having a turbidity corresponding to 1,000,000,000 bacteria per c.c., would be approximately

$$\frac{10 \times 15 \times 0.02}{10 \times 2 \times 0.0002} \times 13 = 9750.$$

Five of the 13 brown colonies were sub-cultured to broth and agglutinated as described. All gave good agglutination with a *B. aertrycke* serum. The figure 9750 was therefore entered unaltered in the records.

It will be noted that the figure taken for the dilution in the turbidity tube is not strictly correct, the quantity added to the total volume with the drops of faecal suspension not being allowed for. The technical error, as will appear later, is so much larger than any error involved by this omission in calculation, that no good purpose would be served by the additional labour involved in using the more accurate figure.

It will be noted also that the actual size of the drops employed is immaterial to the final figure obtained, provided it be kept constant during the whole operation.

Before considering the way in which this and similar figures should be treated in further calculations or arguments, it is essential to determine the errors to which they are liable. These unfortunately are very large, and quite indeterminable in any given case.

That this should be so is not surprising. We are not dealing with a simple bacterial suspension, but with a mixture containing enormous numbers of living and dead bacteria and other organisms of various kinds, and with unknown substances in solution or suspension which may be exerting potent but indeterminate effects on the particular bacteria we are trying to enumerate. Moreover the crowding difficulty, referred to in detail by Wilson, is here unavoidable. We are trying to count viable bacteria of one particular species, which are occasionally present in almost pure culture, are more often mixed with large numbers of viable organisms of quite other kinds, and in many cases form a tiny minority of all the organisms present.

But quite apart from the question of overcrowding, or actual crowding out of the organisms we are trying to isolate, there is another factor which needs consideration. We are relying on one particular phenomenon, the production of hydrogen sulphide and its subsequent interaction with the lead salt, to identify the colonies of *B. aertrycke*. Do such factors as overcrowding of *B. aertrycke* itself, or the crowding of the cultures with organisms of other types, modify this activity in such a way that the characteristic reaction may be suppressed, and colonies of *B. aertrycke* be missed?

If the crowding of other organisms masked the typical reaction, then we might reasonably expect that, if we were to take a considerable number of tubes, which did not show brown colonies but in which there was a chance of colonies of *B. aertrycke* having developed, and if from these tubes we were to sub-culture a series of colonies which, apart from the absence of browning,

might pass for colonies of this organism, we should find on applying agglutination tests that a proportion of such colonies would prove to be *B. aertrycke*.

We have sub-cultured in this way 620 colonies from 124 tubes, none of which showed any brown colonies, and all of which had been inoculated with faeces from mice fed on *B. aertrycke*. Subsequent agglutination tests were negative with each of the 620 cultures.

The question of the effect of overcrowding, where colonies of *B. aertrycke* are present, can clearly be studied by picking non-brown colonies from tubes in which brown colonies occur. Table I shows the result of such a proceeding. It may be noted that the brown colonies were in all cases proved to be *B. aertrycke* by the agglutination of broth sub-cultures.

Table I.

No. of brown colonies	No. of non-brown colonies agglutinating as		No. of brown colonies	No. of non-brown colonies agglutinating as	
	Positive	Negative		Positive	Negative
156	3	2	14	1	9
115	6	4	14	0	5
74	2	3	13	0	5
69	1	4	9	3	2
51	4	6	8	1	9
50	0	7	7	3	2
34	0	5	5	0	3
19	2	8	5	0	5

The answer is perfectly definite. The error due to the failure of browning in some of the colonies of *B. aertrycke*, in tubes where *B. aertrycke* is present and is producing brown colonies, is a very serious one; and it is increasingly serious with increase in the total number of brown colonies present. If we take the cases in which the brown colonies counted numbered 50 or over, we find that, of 42 non-brown colonies tested, 16 or 38.6 per cent. were actually *B. aertrycke*. If we take those cases in which the total brown colonies per tube numbered less than 50, we find that of 63 non-brown colonies tested by agglutination 10, or 15.9 per cent., were actually *B. aertrycke*.

There is no possibility of applying any correction in such cases, since we have no figure for the total non-brown colonies, nor could such a figure be obtained. To enumerate in each tube the colonies which, while not brown, might pass in other respects as colonies of *B. aertrycke* would be an impossible task.

It may reasonably be held that Table I displays the results in too unfavourable a light. The tubes examined in this way were purposely selected as showing relatively few brown colonies, compared to the total colonies present, or alternatively large numbers of brown colonies, sufficient in themselves to cause some degree of crowding. If similar estimations were made on a large sample of the tubes actually utilised for counting, the errors from this failure of browning in colonies of *B. aertrycke* would probably not be large, but the technique will always show a large and unknown error in defect, where the figure obtained is high.

It seems then that the technique adopted will enable us to detect small numbers of *B. aertrycke*, when these are present, with an accuracy at least equal to that obtained by any ordinary method of isolation of such organisms from the faeces. We have little doubt that the accuracy of detection is, in fact, considerably higher than is obtained in any of the ordinary plating methods, if only because of the three widely separated dilutions which are employed. In a considerable proportion of the counts, colonies of *B. aertrycke* have been found on one of the three tubes and not on the other two, and the tube in which they are present is by no means always that containing the largest amount of faecal suspension.

On the other hand the actual figures obtained are always liable to error, and the probable error increases rapidly as the count itself increases. Apart from any other considerations, therefore, we should hesitate to pay too much attention to the actual figure obtained in any one count.

In actual practice, very high figures, over 10,000,000 for instance, may be obtained for the count of viable *B. aertrycke* per c.c. of a faecal suspension of standard turbidity, and the question arises as to how we should treat such figures in considering the numerical results obtained from a considerable series of determinations. It is obvious that there are the gravest objections to the inclusion of such figures in a series in which average values are to be considered. In a series of 50 counts, for instance, which included one count of 10,000,000 it would be quite immaterial, so far as average results are concerned, whether 40 of the remaining 49 counts gave figures of 100 apiece, or whether all were negative.

This overwhelming effect of single large counts appears to us to be so obviously undesirable, when the biological possibilities are kept in mind, that we have thought it best to adopt a purely arbitrary method of recording and charting the results of our counts. This method actually consists in using a logarithmic scale. It should, however, be regarded merely as an arbitrary method of scoring the rate of faecal excretion of any given mouse at any given moment. Any figure greater than 0 and not greater than 10 is scored as 1; any figure greater than 10 but not greater than 100 is scored as 2; any figure greater than 100 but not greater than 1000 is scored as 3, and so on. A count of 10,000,000 would thus be scored as 7, while a count of 10,000,010 would be scored as 8.

When dealing with any considerable number of counts this procedure simply means that we are adopting an arbitrary series of class-intervals, and are using the class-frequencies, instead of the absolute or average numerical values of the individual determinations, in subsequent arguments.

It may, perhaps, be doubted whether quantitative results with so wide a margin of error have any real advantage over simple records of presence or absence of *B. aertrycke*. We shall deal more especially with this question in later reports, where we hope to consider the effect of variations in the distribution of the excretion of *B. aertrycke* among the members of a population,

in which this infection is spreading. For the moment, we will content ourselves with pointing out that the time expended in obtaining roughly quantitative results is very little in excess of that needed for qualitative determinations. With the help of one assistant, two workers can easily put up the necessary dilutions, add the media and roll the tubes, at the rate of 80 specimens an hour. The time taken in preparing the dilution tubes and the suspensions is not great, and the counting is rapidly accomplished, unless an unusually high proportion of the specimens give brown colonies. We doubt if any qualitative method, giving equally accurate results as regards detection, would take less time.

Although it is necessary to confirm the nature of all brown colonies by agglutination tests, and indeed these tests are eminently desirable for quite other reasons which are referred to in a subsequent paper, yet it may be pointed out that the work involved in eliminating brown colonies which are not *B. aertrycke* is not very considerable.

In going over records of the testing of 3795 brown colonies, we find that 2786 of these proved to be *B. aertrycke*; 77 gave negative agglutination results, although other colonies from the same tubes reacted positively; while 932 colonies gave negative results, and no colonies of *B. aertrycke* were identified in the tubes from which they came. It may be noted that 345 of these 932 colonies came from five mice which persistently excreted organisms, other than *B. aertrycke*, which gave rise to brown colonies. We have tested the fermentation reactions of large numbers of such colonies, but the only organisms so far encountered have been *B. proteus*, and an organism which has given the more important reactions of *B. coli communior*, but which appears to be very rarely present in mouse faeces.

We may note, at this stage, the results of examining specimens of faeces from our normal stock.

We have so far examined 692 specimens obtained from 632 normal mice. Of these, 691 specimens were negative, while one specimen gave colonies of *B. gaertner*. *B. aertrycke* was never isolated. These results are in close agreement with those cited by Webster (1923 a). It may be added that brown colonies of any kind were exceedingly rare, being noted in less than 0.5 per cent. of all the specimens examined from normal mice.

In conclusion, we would note a few of the results obtained by repeated examinations of the faeces of individual mice, which have been fed on cultures of *B. aertrycke* on a single occasion. In such cases we have taken counts on the second day following feeding, and on the one or two succeeding days; three counts have been made during each of the two succeeding weeks, and two counts during each of the three weeks following. We have ended our period of observation by making two successive counts on the 41st and 42nd day. These time-intervals have been modified slightly in individual cases, but have not been departed from in any essential respect, except where a mouse has died during the period of examination, or has been kept alive beyond it for some special purpose.

In charting our results we have recorded time-intervals as abscissae and the score of the *B. aertrycke* counts as ordinates. The area between the curve of excretion and the base-line has been blackened to increase clearness of representation. The actual days of observation have been marked below the base-line in the case of each individual mouse.

In Chart I are shown the records of six mice.

Mouse *A* affords a good example of persistent carriage and excretion lasting over several months and then ceasing, the animal remaining in apparently perfect health.

Mouse *B* affords a good example of persistent excretion followed by death from typical enteric infection.

Mouse *C* illustrates a common type of result in which massive excretion for a few days is followed by death with typical lesions.

Mouse *D* shows a not uncommon result in which a mouse excretes *B. aertrycke* on a single occasion only, immediately preceding death from enteric infection.

Mouse *E* shows that an animal may die of typical enteric infection without ever excreting the specific organism in its faeces.

Mouse *F* is an example of the numerous occasions on which administration of *B. aertrycke* by the mouth has been followed neither by the appearance of this organism in the faeces, nor by any other evidence of infection.

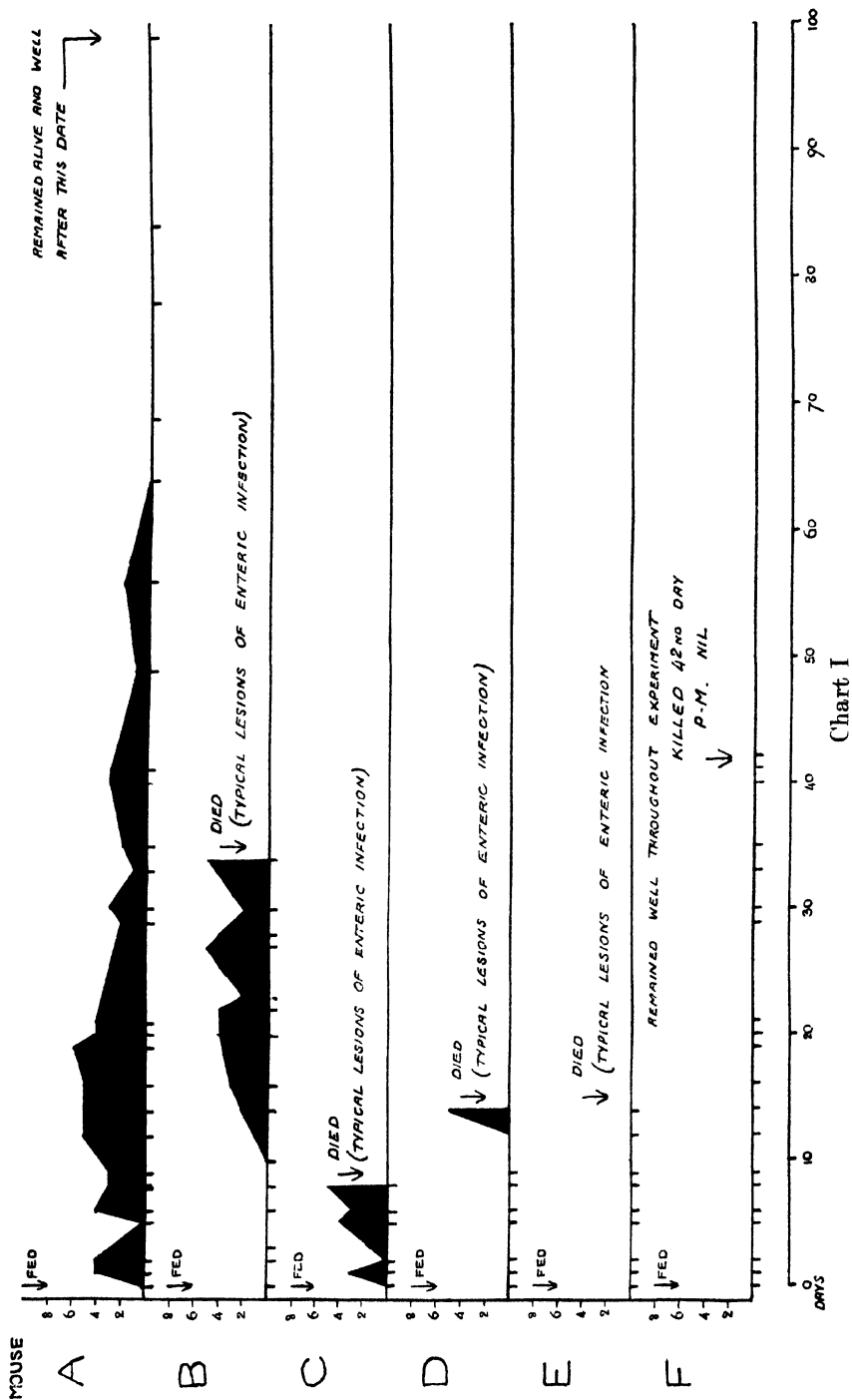
Results of each type are discussed more fully in the succeeding papers. We include them here partly as an illustration of our methods of recording results, partly because it seems to us that they afford in themselves some evidence that the technique is reliable within certain limits. We must avoid the circular argument of approving the technique from a consideration of the results, and then accepting the results because of our approval of the technique; but we may legitimately attach importance to the fact that the results, in a considerable series of observations, tend to be consistent among themselves, and that the general picture presented by our records fits in with our general knowledge of the processes of infection.

APPENDIX.

The composition of the medium employed for the bacterial counts is as follows:

Beef extract (Lemco)	5 gms.	Salicin	10 gms.
Peptone (Witte)	... 10 „	Andrade's Indicator	...		10 c.c.
Sodium chloride	... 5 „	Agar	25 gms.
Lactose	... 10 „	Water	1000 c.c.
Saccharose	... 10 „				

The beef extract, peptone and sodium chloride are added to the water in a flask and placed in the steamer till dissolved. The agar is then added and the flask replaced in the steamer till this has dissolved, when it is again removed and white of egg is added for clearing. This is coagulated in the



steamer and the medium is then filtered. The pH is determined and is adjusted to 7.1. The Andrade indicator is then added and the medium is autoclaved at 107° C. for 20 minutes. Meanwhile the requisite amount of lactose, saccharose and salicin have been dissolved in the minimal amount of distilled water (about 30 c.c.) and placed in the steamer for 20 minutes. As soon as the medium is removed from the autoclave this concentrated solution is added to it, and stirred with a sterile rod. The medium is immediately transferred to small flasks and tubes, with due precautions as regards sterility and these flasks or tubes are then placed in the steamer for 10 minutes, after which they are stored until required. At this stage the pH of the medium is again determined, and should be found to be in the near neighbourhood of 7.4.

When the medium is to be employed the necessary amount is melted in the steamer and placed in a water-bath at 55° C. A solution of lead acetate, approximately 1 per cent., is prepared by taking a small flask containing 30 c.c. of sterile distilled water, raising this rapidly to the boil and boiling for 2 minutes. The loose wool-plug is then removed and 0.3 gm. of neutral lead acetate (lead diacetate), previously weighed out and kept in a small sterile tube, is dropped into the flask; the wool-plug is at once replaced. This procedure ensures the removal of the dissolved CO₂ and prevents the precipitation of lead carbonate. The solution so prepared is at once added to the melted medium, by means of a sterile pipette, in the proportion of 5 c.c. for every 100 c.c. of medium, and the flask or tube is quickly and vigorously shaken. With a separate sterile pipette 5 c.c. of a sterile 1 per cent. solution of disodium hydrogen phosphate is added, for every 100 c.c. of medium, and the flask or tube is again shaken, and is then placed in the water-bath at 55° C. The medium is now ready for addition to the culture tubes.

The addition of the lactose, saccharose and salicin to the medium is probably optional, though the presence of small amounts of some fermentable carbohydrate is certainly not a matter of indifference. The presence of these fermentable substances, however, sometimes yields useful information with regard to the general character of the bacteria present¹.

What the exact effect may be of the addition of sodium phosphate in the last stage of preparation, we are not in a position to say. The exact change which occurs in the complex agar medium from the addition of the lead salt is at present unknown. We were led to test the effect of the addition of the phosphate for reasons which need not be entered into here. As a matter of experience we have never had any difficulty with this medium since we made this addition to our previous procedure.

¹ In recent experiments we have replaced the lactose, saccharose and salicin by dextrose (1%), since this gives better results when *B. aertrycke* greatly outnumbers other organisms in the faeces.

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THE EXCRETION OF *B. ENTERITIDIS* (*AERTRYCKE*) IN THE FAECES OF MICE AFTER ADMINISTRATION BY MOUTH.

BY W. W. C. TOPLEY AND JOYCE AYRTON.

(From the Department of Bacteriology and Preventive Medicine,
University of Manchester.)

A Report to the Medical Research Council.

(With 10 Charts and 1 Text-figure.)

IN the preceding report (Topley and Ayrton, 1923 *a*) we have described a technique by which measurements may be obtained of the excretion of *B. enteritidis* (*aertrycke*)¹ in the faeces of mice.

For reasons which have been set out in the report referred to above, we have adopted an arbitrary method of scoring the number of viable *B. aertrycke* present in any given specimen of faeces, using a series of class-intervals which increase in extent in a geometric ratio, and assigning the same score to all results which fall within the same class-interval.

In the present report we describe the results of a considerable series of experiments in which we employed this technique in the endeavour to obtain an answer to the two following questions:

(1) Do different strains of *B. aertrycke* vary in the manner in which they are excreted in the faeces of mice, following administration by the mouth?

(2) What, if any, is the relation between the dose of viable bacilli administered, and the frequency, amount or persistence of the subsequent faecal excretion?

The results obtained in studying the first of these two questions were so striking, that the investigation has largely concerned itself with the points raised in this part of the work. The question of dosage has, however, never been lost sight of, and all the available data are considered in the latter part of this report.

GENERAL TECHNIQUE.

The technique which has been adopted in the experiments under consideration is as follows:

In the majority of cases one feeding only of bacterial culture is administered to each batch of mice. In each experiment ten mice are fed. A preliminary examination of the faeces of each mouse is carried out to determine the absence of *B. aertrycke*. To each mouse we then administer 0.1 c.c. of an

¹ The organism referred to is of the "Mutton" variety. For convenience it will in future be referred to simply as *B. aertrycke*.

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ADDENDUM

To the paper published in *Journal of Hygiene*, XXII, pp. 234—263.

The results of experiments, carried out since this report was submitted for publication and the proofs corrected, have led us to alter our views with regard to the nature of those cultures of *B. aertrycke*, which are agglutinated with both type and group antisera.

Conclusions 1—3, and the latter part of Conclusion 7, in the form set out in the report, cannot therefore be accepted.

The questions at issue are being re-investigated in the light of the fresh evidence obtained.

18 hours' broth culture of *B. aertrycke*, or a dilution of it in nutrient broth, by allowing five drops of the fluid to fall into the opened mouth of the mouse from a dropping pipette, calibrated to deliver 50 drops per c.c. An interval of a few minutes is allowed to elapse between giving the first three drops and the last two, to render swallowing easier and more certain. This method is more accurate than administration by feeding on bread soaked in bacterial culture, and it appears to us that it must give a more exact representation of the course of events in the natural spread of infection than is obtained by intra-stomachal injection through a catheter, as in the method employed by Webster (1922).

To each mouse, in each experiment, we have given five drops of culture. In the case of the first pair, in each batch of ten, the culture is undiluted: in the case of the second pair it is diluted ten times: in the case of the third pair one hundred times, and so on. In each series, therefore, the actual doses administered are as follows:

Mice	1 and 2	...	0.1 c.c. of an 18 hours' broth culture		
"	3 "	4	...	0.01	" "
"	5 "	6	...	0.001	" "
"	7 "	8	...	0.0001	" "
"	9 "	10	...	0.00001	" "

In all experiments each mouse is kept throughout in a separate cage, so that there is no opportunity for passage of bacilli from mouse to mouse. Each batch of mice is observed for 42 days, the faeces being examined on the second and third days after feeding, three times during each of the following two weeks, twice during each of the next three weeks, and on the 41st and 42nd days. In some cases more frequent examinations have been made.

Any mouse which dies is examined post-mortem, according to the routine procedure which has already been described (Topley, 1922). On the 42nd day all surviving mice are killed, with a few exceptions in which an individual mouse is retained for some special reason. A post-mortem examination is made, and any abnormality noted. A portion of the spleen is excised and dropped into a tube of broth, which is incubated for two days at 37° C. before being discarded as sterile. If any growth occurs, sub-cultures are made on plates of McConkey's medium.

In all cases in which colonies are obtained, whether from faeces, from the tissues of mice dying from enteric infection, or from the spleen cultures from survivors, which on general grounds are regarded as probably *B. aertrycke*, such colonies are sub-cultured to broth, killed by the addition of formalin followed by heat, and tested against high-titre agglutinating sera, as will be referred to in more detail later.

In the case of each mouse, therefore, we may find evidence of infection, using this term in its broadest sense, in three distinct ways. We may isolate *B. aertrycke* from the faeces: the mouse may die of *B. aertrycke* infection or, dying from some intercurrent disease may yield cultures of *B. aertrycke* from

its tissues: or it may remain in apparent health until it is killed after 42 days, but from its spleen, removed after death, *B. aertrycke* may be isolated.

It will be convenient, as regards the question of bacterial variation, to state at once the general conclusions arrived at, and then to produce our evidence, describing a few experiments in detail and finally summarising the results of the whole series.

Our results would seem to show that:

(a) Wide variations occur in the behaviour of different strains of *B. aertrycke*, as judged by the excretion of this organism in the faeces of mice, after it has been administered by the mouth.

(b) These variations are discontinuous. Certain strains are excreted in the faeces of a high proportion of the mice to which they are fed, and this excretion may persist over considerable periods of time. Other strains are either not excreted at all, or they are excreted by few of the mice to which they are fed, and then the excretion is transient. The feature which distinguishes sharply between the two kinds of strain is the capacity for giving rise to persistent excretion. Where such capacity is demonstrated the strain in question always belongs to a given variety, which may be recognised in other ways. Where such a capacity cannot be demonstrated, in so limited a series of mice as the ten employed in each of these experiments, it is not safe to assume that the strain in question does not belong to this variety, although the probability is strongly against its doing so. The contrasted variety never gives rise to persistent excretion, so far as can be recognised by the technique employed.

(c) The strains which are persistently excreted are those which are agglutinated with a "group" anti-serum, using this term in the sense attached to it by Andrewes (1922). They may also react to a "type" or monospecific antiserum. It follows that they always contain group antigen, and may or may not contain type antigen.

(d) The strains which are not so excreted are those in which group antigen is absent. They contain the type antigen alone.

(e) These results hold true both for smooth and for rough varieties of *B. aertrycke*; but it is much less common to get persistent excretion of a rough than of a smooth strain. Where persistent excretion of a rough strain does occur, the strain always possesses group antigen.

It will be well first to consider, very briefly, the relevant facts as regards the serological relationships of *B. aertrycke*. It will suffice, for the moment, to recall the crucial results obtained by Andrewes (1922).

Following a line of reasoning which is fully set out in the paper referred to, Andrewes made use of two kinds of agglutinating sera. One of these was strictly specific. It was prepared by thoroughly absorbing a high-titre aertrycke serum with closely related organisms, such as *B. paratyphosus* B which were themselves agglutinated by this serum in its untreated state. The other was a paratyphosus B serum which *ex hypothesi* did not contain the specific or type

agglutinin, but which was known to give group agglutination with *B. aertrycke* in high dilution.

In this way, Andrewes was armed with one reagent which would pick out type antigen, and another which would pick out group antigen. He found, when numerous colonies of a given strain of *B. aertrycke* were subcultured into broth and these broth cultures were killed by the addition of formalin and tested against these monospecific and group sera, that the strains so isolated fell sharply into two classes, the one agglutinating to titre, or almost to titre, with the type serum, and failing to react with the group serum, the other behaving in exactly the opposite way. With some strains, however, there was slight cross agglutination, while very occasionally Andrewes met with strains which agglutinated well with both sera. The striking fact stood out clearly, that from any given culture of *B. aertrycke* two kinds of strain could be separated, one reacting with type antiserum, the other with group antiserum.

It was, however, evident in all Andrewes' work that these type and group varieties were markedly unstable in culture. One variety might change into the other with startling and disconcerting rapidity. There was, it should be emphasised, no question of a variation which would transgress the limits recognised as demarcating one species or type from another. *B. aertrycke* never changed into *B. paratyphosus* B, nor *vice versa*. It was impossible to differentiate a group strain of *B. aertrycke* from a group strain of *B. paratyphosus* B by agglutination, but when either of these group strains gave rise to type variants, these were always *B. aertrycke* or *B. paratyphosus* B as the case might be. Thus it seemed clear that the group variety of *B. aertrycke*, for instance, always possessed a minimal amount of type antigen. There was, at least, some controlling factor which ensured that, when the type antigen did appear in detectable amounts, it was always of the *aertrycke* type and never of any other.

We may say at once that our work has confirmed the results obtained by Andrewes in all important respects. There are two slight exceptions. We have met, far more frequently than he appears to have done, strains of *B. aertrycke* which react equally well with type and group antisera. We consider these more fully in a further report (Topley and Ayrton, 1924¹) and for the moment we would merely state that we should be disposed to recognise at least three varieties of *B. aertrycke*, (a) type strains, (b) group strains, and (c) mixed strains.

We have on several occasions met with strains which appear to have lost all agglutinability. These latter have played no part in the present series of experiments, and we shall not, for the moment, consider them further.

As mentioned in the preceding report, in every case in which brown colonies were encountered in making a bacterial count on a specimen of faeces, indicating the formation of hydrogen sulphide, a selection of these colonies were subcultured to broth and tested against high-titre agglutinating

¹ To appear in *Journal of Hygiene*, XXII. No. 3.

sera. In all but the first four of the experiments here reported the sera employed were (a) a high-titre mono-specific (absorbed) aertrycke serum, (b) a high-titre newport serum, known to agglutinate group strains of *B. aertrycke*, and (c) a high-titre gaertner serum, also known to agglutinate some group strains of *B. aertrycke*.

In this way it was possible to divide all strains isolated from the faeces into type, group or mixed strains, on the basis of the agglutination results. All strains isolated from the tissues were tested in an identical manner.

In the first four experiments, the aertrycke serum employed had not been absorbed. In these experiments, therefore, we are only justified in dividing the strains into two classes: (a) type strains, and (b) group or mixed strains. Due allowance is made for this in all tables, etc.

VARIABILITY OF STRAINS IN CULTURE.

The thesis which we consider to be supported by our results relates a particular type of agglutination reaction to a particular type of behaviour as regards excretion. It is clear that, for this argument to be valid, we must place a reasonable degree of reliance on the results of our agglutination tests, in the sense that we can judge from them the kind of organisms that were present in the faeces.

In this connection we would cite the following facts.

It is no unusual event to isolate, from the faeces of a given mouse, a series of strains of *B. aertrycke* of one serological type, and one only. For instance, from the faeces of five mice in one experiment, 58 strains of *B. aertrycke* were isolated. All agglutinated to titre with a group antiserum, but none gave any agglutination with a type antiserum. Results of this kind have been of frequent occurrence. Indeed it has been very unusual to isolate pure group and pure type varieties of *B. aertrycke* from the same mouse or series of mice, except in a few cases where mixed cultures have purposely been administered. We may also note the fact that approximately 93 per cent. of all strains of *B. aertrycke* isolated from the faeces have reacted with a group antiserum. Such results could not be the consequence of spontaneous variations in sub-culture, if such variations proceeded with equal readiness in either direction. They could only be explained, on such a basis, by supposing that the change from type to group occurred very frequently, while the change in the opposite direction occurred very rarely.

If such were the case, we should not expect, in any series of experiments, to obtain a preponderance of strains reacting with the type antigen alone. In three experiments, in which the excretion of *B. aertrycke* was very scanty, we isolated 54 strains of this organism from the faeces. Fifty-one of these strains, or 94 per cent., agglutinated with a type antiserum but not with a group antiserum.

Finally, we would cite certain experiments, referred to in a later report (Topley and Ayrton¹), in which we inoculated cultures of *B. aertrycke*

¹ See footnote, p. 237.

intraperitoneally into mice, and tested by agglutination numerous strains recovered from the tissues after death. Two series, each of ten mice, were inoculated with strains believed to be of the pure type variety. From the tissues of the mice which died, or were killed, 382 strains of *B. aertrycke* were recovered. Of these, 330 agglutinated with a type serum only, 8 agglutinated with a group serum only, and 44 agglutinated with both sera. Two other series of ten mice each were inoculated with strains believed to be of the pure group variety. From the tissues of mice which died or were killed 313 strains of *B. aertrycke* were isolated. Of these, 231 agglutinated with a group serum only, one agglutinated with a type serum only, and 81 agglutinated with both sera.

It seems probable that the spontaneous variation, which undoubtedly occurs in broth cultures, is not of such a nature as seriously to obscure the true state of affairs, with regard to the distribution of type and group varieties in the tissues or excretions from which the cultures were obtained.

Turning now to the question of our control over the cultures which we administer by mouth, the position is much less satisfactory. We can determine the nature of such cultures only by testing a sample at the shortest possible interval before administration, and here we encounter a formidable difficulty.

We desire to know the nature of the viable bacilli in the culture we are administering, but our test sample will tell us only the distribution of the two kinds of antigen among all bacilli in the culture, the living and the dead. It is probable that, if a relatively small proportion of all the bacilli were of a different variety from that we desire to administer, we should still detect them by agglutination against high-titre sera. It is easy to show, however, that this does not apply when we consider the viable bacilli alone.

A flask of broth was inoculated from a culture of *B. aertrycke*, which we believed to contain only the type variety, and was then incubated at 25° C. After various intervals a sample of the culture was withdrawn, killed with formalin and heat, and agglutinated. At the same time a plate culture was prepared. Next day 50 colonies were picked from this plate, subcultured into broth, grown for 18 hours, at 25° C., killed and agglutinated. Thus we had a record of the agglutination reaction of the original culture after various periods of incubation, while the results obtained with the colonies from the plates gave us a picture of the nature of the viable portion of the bacillary population at the same moment.

Without giving detailed results, it is sufficient to record that after six hours the original culture agglutinated with a type serum alone, while from a plate prepared at this time we isolated 43 colonies of type bacilli and 7 mixed. On another occasion, when the original culture still responded only to the type antiserum, 17 of the 50 colonies subcultured from the corresponding plate reacted with both type and group antisera.

EXPERIMENTAL RESULTS.

The striking difference between the quantitative excretion of *B. aertrycke*, which followed the administration to mice of different strains of this organism, was noted very early in the course of our investigation. The routine examination of a selection of all strains, by testing them against the three agglutinating sera, at once brought to our notice the fact that the great majority of these strains were agglutinated by the group serum, and that strains which were agglutinated by the type serum alone were very rarely isolated from the faeces. Past experience had shown us that such strains were quite common in cultures obtained from the tissues during epidemics of enteric infection in mice.

In all succeeding experiments, therefore, an attempt was made to administer a strain of known serological variety. Colonies were picked from a plate culture, obtained from a growth in broth which had itself responded to agglutination by showing purely type or purely group affinities. Six to twelve of these colonies were subcultured to broth tubes containing 10 c.c. of medium. These tubes were incubated aerobically at 22-25° C., or anaerobically at 37° C. Very numerous tests had shown us that, under either of these conditions, there was less probability of spontaneous variation than if the cultures were incubated aerobically at 37° C. As the result of a limited number of experiments we were led to the view that anaerobic incubation had a very definite effect in retarding these variations, but more extended experience has led us to doubt whether anaerobiosis acts in any other way than in slowing the rate of bacterial multiplication, though it has proved to be one of the most certain methods of inhibiting a rapid change from one serological variety to another.

After 16 to 17 hours' incubation the tubes were removed from the incubator, a sample was taken from each of them, killed by formalin and heat and tested against the three sera employed throughout the investigation. Any culture which gave indication of possessing both type and group antigen was discarded, and one of the cultures which were sharply differentiated as belonging to the variety desired was employed for feeding purposes.

Other technical details, concerning methods of administration, dosage, etc., have been referred to above and need not be repeated. The methods employed in recording and charting the results may however be briefly recapitulated.

The actual bacterial counts are first expressed as numbers of viable *B. aertrycke* per c.c. of a faecal suspension of a turbidity corresponding to a bacterial content of 1,000,000,000 per c.c. Any count not greater than 10 is scored as 1, any count greater than 10 but not greater than 100 is scored as 2 and so on, using in all cases the index or logarithm of the first integral power of 10 greater than the actual count recorded. The reasons for adopting this convention have been discussed in a previous report (Topley and Ayrton, 1923^a).

In the charts, the results for each mouse are recorded along a separate base-line. A curve of excretion is drawn, using the figures scored for the bacterial counts in the manner described above. The area between this curve and the base-line is blackened to increase the clearness of representation. A time-scale is placed below the whole series of curves, and also on an upper base-line. Along each base-line, corresponding to an individual mouse, are recorded the days on which bacterial counts of the faeces were carried out. The record of each individual mouse terminates in an arrow pointing upwards. Where these arrows indicate deaths occurring during the course of the experiment, a corresponding square will be found on the upper base-line. This is black if a post-mortem examination proved the death to be due to enteric infection. It is unshaded if no evidence of such infection could be obtained. On the last day of each experiment, those mice which remained alive were killed, and each mouse is represented by an upward pointing arrow with a circle at its lower extremity. A post-mortem examination was carried out on each mouse, and cultures were prepared from the spleen. If any given spleen culture yielded a growth of *B. aertrycke*, the circle beneath the corresponding arrow is blackened. If the spleen culture proved negative the circle is left unshaded.

One other figure, which is entered in the tables and in the records of those experiments which are referred to in more detail, needs an explanation. It is recorded as the "excretion coefficient." It was clearly desirable to adopt some numerical expression as a measure of the total excretion during any given experiment of any mouse, or group of mice. One very simple method of arriving at such an expression is to record the percentage of all specimens of faeces examined from such a group, which gave positive results. We are not, at the present time, prepared to maintain that this measure is in any way inferior to the one we have adopted. So far as the present enquiry is concerned it gives almost the same relative results. It does not, however, take into account the number of bacilli excreted. It does not differentiate between a specimen of faeces containing viable *B. aertrycke* in such numbers as to give an almost pure culture, and another specimen in which they form a tiny minority of the viable bacteria present.

We have therefore adopted a formula which takes account of the relative number of viable *B. aertrycke* in the positive specimens. In arriving at the excretion coefficient for any group of mice, we have added together the scores obtained by all specimens of faeces examined, divided the sum by the total number of specimens examined and multiplied the resulting figure by 100. The coefficient thus expresses the total score which would be obtained in examining 100 specimens of faeces, assuming that the distribution of *B. aertrycke* was the same as that actually observed.

The following experiments will serve as examples of the whole series. We have simply recorded the results obtained without textual description.

Exp. D. Chart I. Ten mice were fed on an 18 hours' broth culture of *B. aertrycke* (smooth group or mixed strain) and observed over 42 days with the following results:

Number of mice fed	10
Number of mice which excreted <i>B. aertrycke</i>	8
Number of specimens of faeces examined	138
Number of specimens of faeces positive	31
Excretion coefficient	74
Number of colonies from faeces tested by agglutination	168
Number of colonies reacting as	Type	0
	Mixed or Group	168
Number of mice which died	4
Number of mice positive on post-mortem examination	3
Number of colonies from the tissues of these mice tested by agglutination	18
Number of colonies reacting as	Type	0
	Mixed or Group	18
Number of survivors killed on 42nd day	6
Number of survivors with positive spleen cultures	3
Number of colonies from these cultures tested by agglutination	9
Number of colonies reacting as	Type	0
	Mixed or Group	9

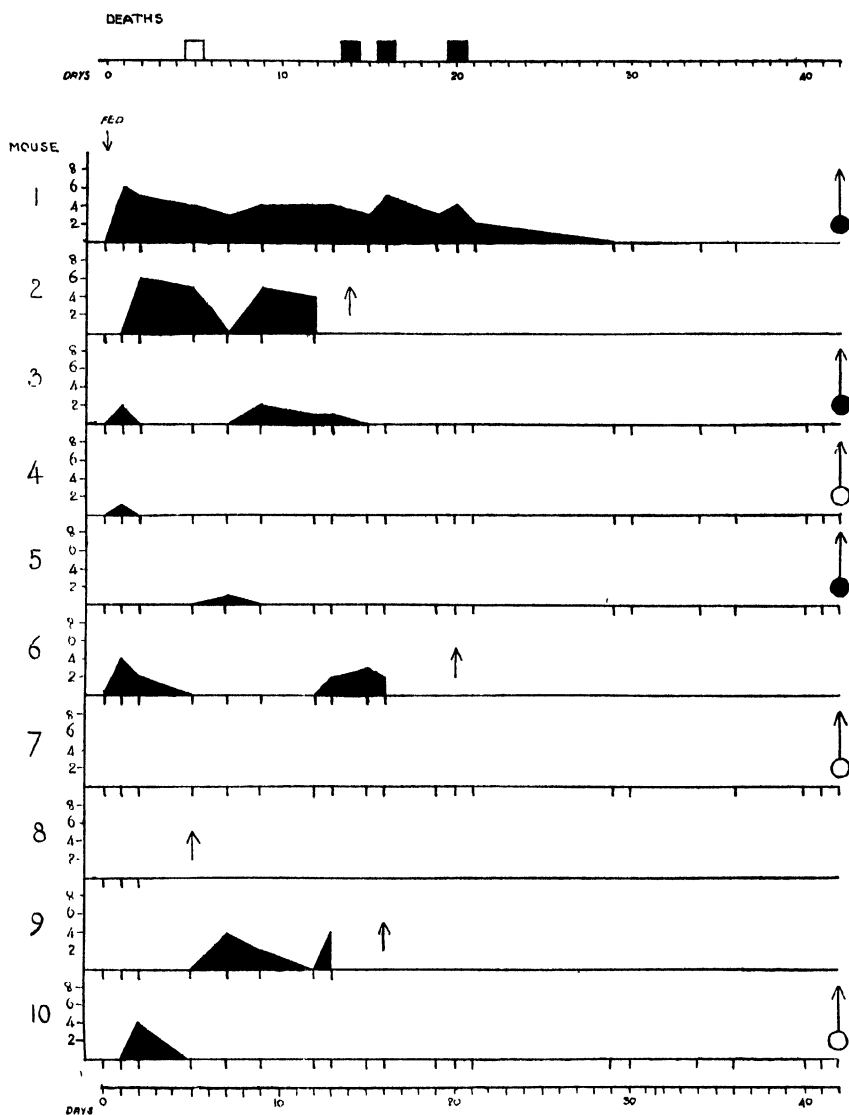
The agglutinating serum used in this experiment did not allow us to differentiate between group and mixed strains. We would call attention to the high proportion of mice excreting *B. aertrycke*, the persistence of the excretion in certain cases, and the fact that all strains isolated contained group antigen. It may also be noted that the examination of those mice which survived, in apparently perfect health, until the 42nd day showed that half of them were harbouring viable *B. aertrycke* in their spleen tissue.

Exp. E. Chart II. Ten mice were fed on an 18 hours' broth culture of *B. aertrycke* (smooth type strain) and observed for 42 days with the following results:

Number of mice fed	10
Number of mice which excreted <i>B. aertrycke</i>	0
Number of specimens of faeces examined	139
Number of specimens of faeces positive	0
Excretion coefficient	0
Number of mice which died	2
Number of mice positive on post-mortem examination	1
Number of colonies from the tissues of this mouse tested by agglutination	20
Number of colonies reacting as	Type	14
	Group	0
	Mixed	6
Number of survivors killed on 42nd day	8
Number of survivors with positive spleen cultures	2
Number of colonies from these cultures tested by agglutination	20
Number of colonies reacting as	Type	7
	Group	0
	Mixed	13

In this experiment the feeding of a pure type culture was followed by the entire absence of faecal excretion; but at least three mice became infected, since one mouse died of typical enteric infection and two mice were found to be harbouring *B. aertrycke* in their spleen tissue after 42 days.

It may be noted that mixed as well as type cultures were isolated from the tissues, yet no mixed strains ever appeared in the faeces.



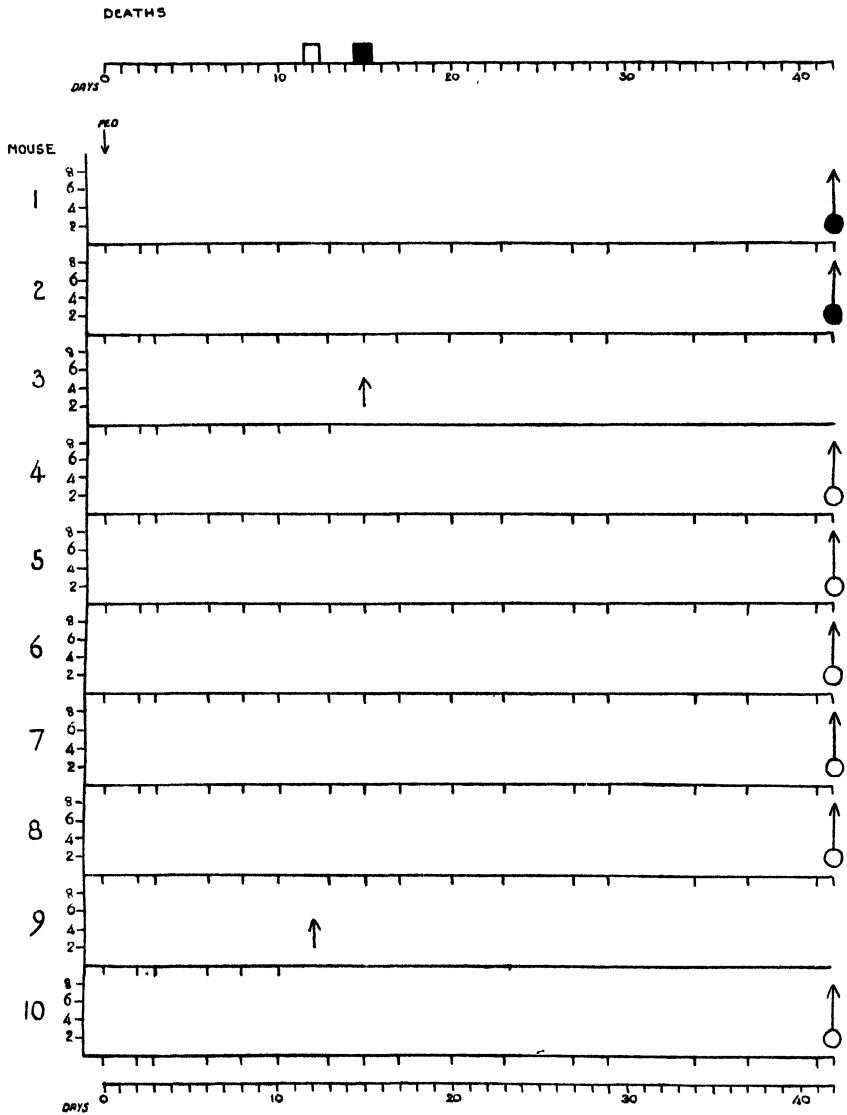


Chart II. Exp. E.

Exp. F. Chart III. Ten mice were fed on an 18 hours' broth culture of *B. aertrycke* (smooth type strain) and observed for 42 days with the following results:

Number of mice fed	10
Number of mice which excreted <i>B. aertrycke</i>	3
Number of specimens of faeces examined	144
Number of specimens of faeces positive	5
Excretion coefficient	13
Number of colonies from faeces tested by agglutination	15
Number of colonies reacting as	{ Type	15
	{ Group	0
	{ Mixed	0
Number of mice which died	2
Number of mice positive on post-mortem examination	2
Number of colonies from the tissues of these mice tested by agglutination	39
Number of colonies reacting as	{ Type	27
	{ Group	0
	{ Mixed	12
Number of mice killed on 42nd day	8
Number of mice with positive spleen cultures	0

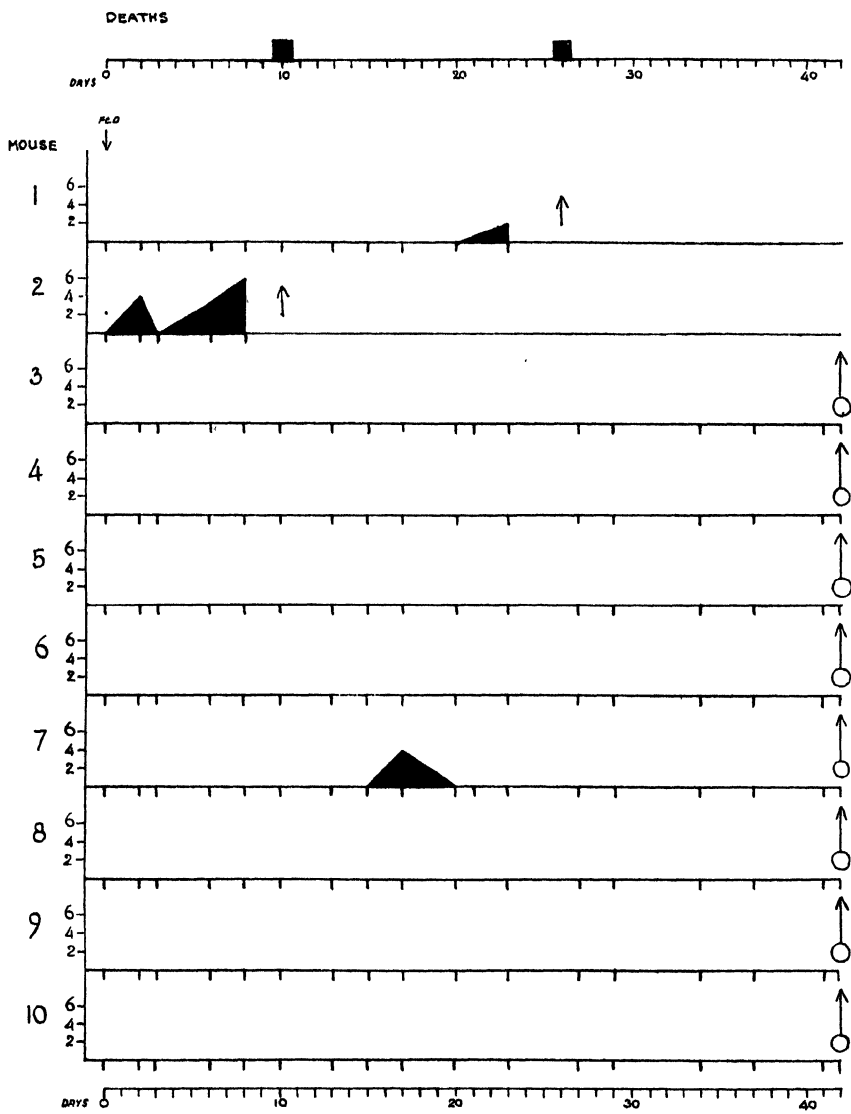
In this experiment there was transient excretion on the part of three mice, and two mice died of typical enteric infection.

Exp. A. Chart IV. This is an example of the failure of an attempt to feed ten mice on a pure type strain. The mice were observed for 42 days with the following results:

Number of mice fed	10
Number of mice which excreted <i>B. aertrycke</i>	6
Number of specimens of faeces examined	143
Number of specimens of faeces positive	56
Excretion coefficient	129
Number of colonies from faeces tested by agglutination	286
Number of colonies reacting as	{ Type	0
	{ Mixed or Group	286
Number of mice which died	3
Number of mice positive on post-mortem examination	2
Number of colonies from the tissues of these mice tested by agglutination	12
Number of colonies reacting as	{ Type	0
	{ Mixed or Group	12
Number of survivors killed on 42nd day	3
Number of survivors with positive spleen cultures	0

Four mice from this experiment were saved alive, in order to test the length of time over which excretion would continue.

This is one of the four experiments in which the sera employed did not differentiate between group and mixed strains. It is of interest because, although we failed to exclude group or mixed bacilli from the culture used for feeding, there can be no reasonable doubt that we administered viable type bacilli. It therefore illustrates the point that a culture containing both type and group bacilli tends to behave, as regards excretion in the faeces, in the same way as a group culture containing group or mixed bacilli only. Thus, even when an experiment in which an attempt to feed a group strain appears to have been entirely successful, we are not justified in assuming that we have administered no type bacilli.



Exp. J. Chart V. Ten mice were fed on a mixture containing equal parts of two cultures of *B. aertrycke*, one a smooth type strain, the other a smooth group strain. The mice were observed for 42 days with the following results:

Number of mice fed	10
Number of mice which excreted <i>B. aertrycke</i>	3
Number of specimens of faeces examined	137
Number of specimens of faeces positive	19
Excretion coefficient	49
Number of colonies from faeces tested by agglutination	76
Number of colonies reacting as	{ Type	8
	{ Group	55
	{ Mixed	13
Number of mice which died	2
Number of mice positive on post-mortem examination	2
Number of colonies from the tissues of these mice tested by agglutination	40
Number of colonies reacting as	{ Type	20
	{ Group	16
	{ Mixed	4
Number of survivors killed on 42nd day	8
Number of survivors with positive spleen cultures	3
Number of colonies from these cultures tested by agglutination	30
Number of colonies reacting as	{ Type	0
	{ Group	2
	{ Mixed	28

As in the last experiment, group or mixed bacilli dominate the situation as regards faecal excretion, but it will be noted that the distribution of type and group strains in the tissues differs from that in the faeces.

All the experiments so far recorded were carried out with smooth strains. In view of the knowledge which we already possess, with regard to the differences in the behaviour of smooth and rough strains when introduced into the tissues, it was natural that we should investigate the behaviour of rough variants when administered by the mouth. The general relation of roughness and smoothness to the other factors investigated is discussed in another report (Topley and Ayrton, 1924¹), but we include the feeding experiments in this paper for the sake of completeness.

The rough variants were obtained by allowing broth cultures of *B. aertrycke* to remain in the incubator at 37° C. for 7 to 10 days or longer, then plating and picking off the rough colonies, which seldom failed to appear in smaller or greater numbers after this period.

It is not necessary to present detailed accounts of experiments in which the rough strains have been employed. The association between the presence of group antigen and excretion in the faeces was as close with the rough strains as with the smooth; but it was equally evident, taking the rough strains as a whole, that excretion in the faeces was less frequent than with the smooth strain. Reference to Tables I, II, III and IV in which the results of all experiments are summarised, will make these points clear.

¹ See footnote, p. 237.

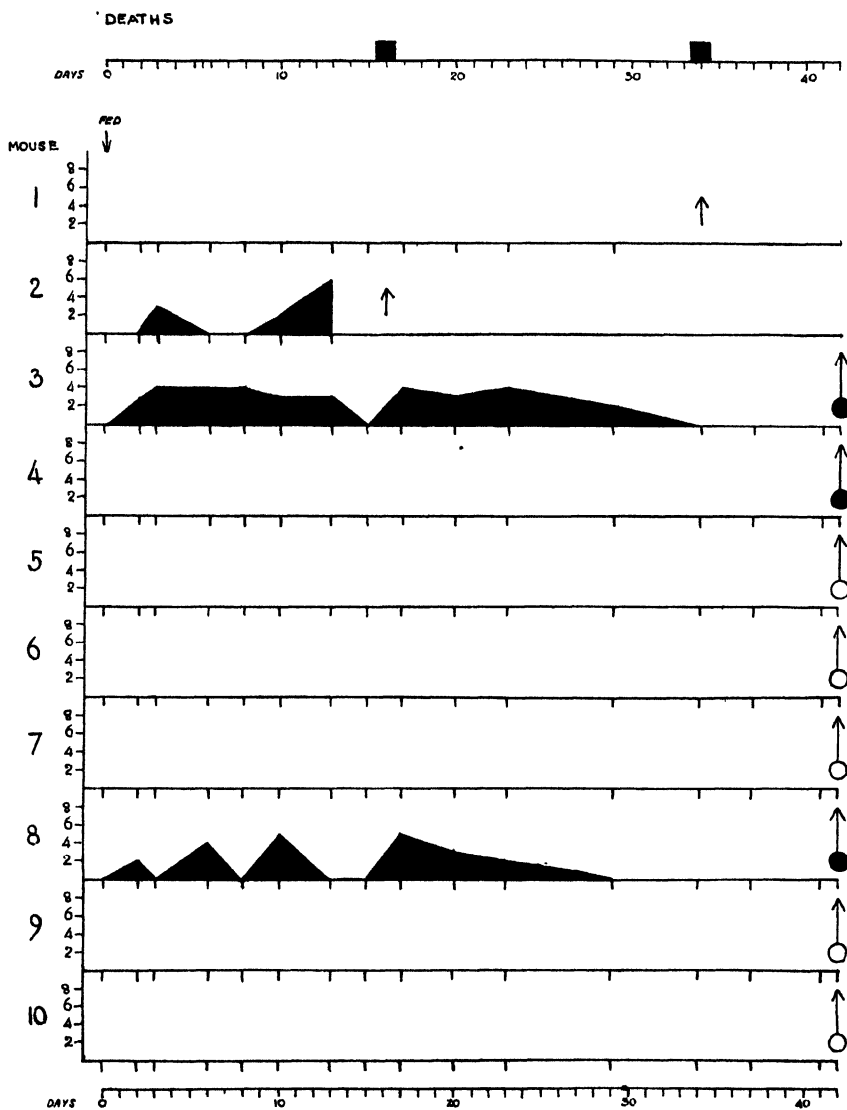


Chart V. Exp. J.

EXPERIMENTS IN WHICH REPEATED ADMINISTRATIONS OF
B. AERTRYCKE WERE CARRIED OUT.

Although the evidence obtained by single feedings of *B. aertrycke* seemed conclusive as regards the main point at issue, it seemed desirable to carry out a series of experiments in which an attempt should be made to establish faecal excretion of *B. aertrycke* by repeated administration by the mouth, using in some experiments cultures which we believed to be pure type strains, in others cultures which we believed to be pure group strains, and in some cases a mixture of the two. We expected that we should fail in our attempt to carry out repeated feedings with pure type strains, for the reasons we have already referred to. In several cases our expectation was realised, but we persisted, in these experiments in feeding type strains, in order to see whether type bacilli would appear in the faeces.

The following experiments will serve as examples:

Exp. Q. Chart VI. Five mice were fed on six occasions with cultures of *B. aertrycke* (smooth group strains). Each mouse, at each feeding, was given 0.02 c.c. of an 18 hours' broth culture of the strain employed. The mice were observed for 42 days, dating from the first feeding, with the following results:

Number of mice fed	5
Number of mice which excreted <i>B. aertrycke</i>	5
Number of specimens of faeces examined	75
Number of specimens of faeces positive	33
Excretion coefficient	155
Number of colonies from faeces tested by agglutination	139
Number of colonies reacting as	{ Type						4
	{ Group						36
	{ Mixed						99
Number of mice which died	0
Number of survivors killed on 42nd day	4
Number of survivors with positive spleen cultures	1
Number of colonies from this culture tested by agglutination	10
Number of colonies reacting as	{ Type						2
	{ Group						0
	{ Mixed						8

One mouse was kept alive for another purpose.

This experiment calls for no special comment.

Exp. W. Chart VII. Five mice were fed on five occasions with cultures of *B. aertrycke* (smooth type strains). The doses given were the same as in *Exp. Q.* The mice were observed for 42 days dating from the first feeding with the following results:

Number of mice fed	5
Number of mice which excreted <i>B. aertrycke</i>	1
Number of specimens of faeces examined	50
Number of specimens of faeces positive	1
Excretion coefficient	8
Number of colonies from faeces tested by agglutination	2
Number of colonies reacting as	{ Type						2
	{ Group						0
	{ Mixed						0

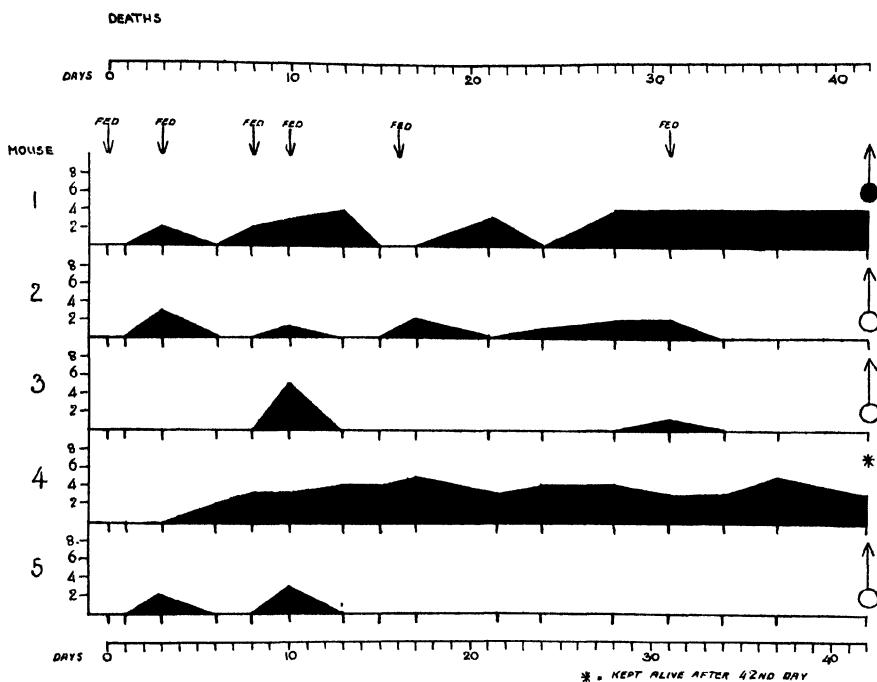


Chart VI. Exp. Q.

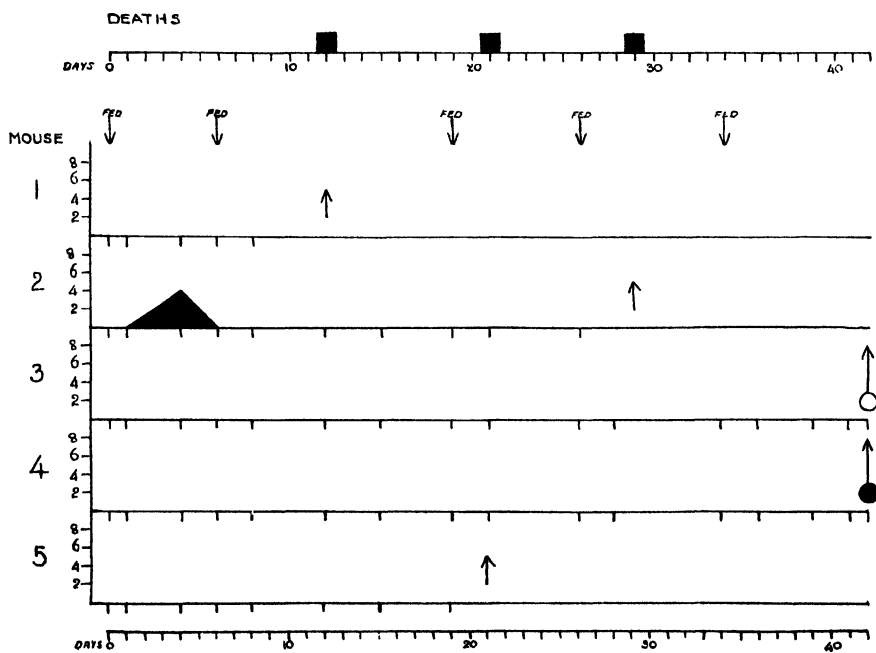


Chart VII. Exp. W.

Number of mice which died	3
Number of mice positive on post-mortem examination	3
Number of colonies from these mice tested by agglutination	44
Number of colonies reacting as { Type	38
Group	0
Mixed	6
Number of survivors killed on 42nd day	2
Number of survivors with positive spleen cultures	1
Number of colonies from this culture tested by agglutination	10
Number of colonies reacting as { Type	2
Group	0
Mixed	8

This experiment may be contrasted with Exp. *Q*. It affords also another illustration of the fact that mice may be infected with type strains of *B. aertrycke*; and die of the infection, without ever excreting *B. aertrycke* in their faeces.

We may take this opportunity of emphasising a point, which must already have become obvious. It is quite clear, that, as a general rule, type bacilli do not change into group or mixed bacilli in the intestine. It is equally clear that, if type bacilli change into group or mixed bacilli in the tissues, the group or mixed bacilli so produced do not find their way into the intestinal tract.

Exp. P. Chart VIII. In this experiment an attempt to carry out six feedings with pure type strains resulted in failure. All details of the technique are the same as Exps. *Q* and *W*, the results were as follows:

Number of mice fed	5
Number of mice which excreted <i>B. aertrycke</i>	5
Number of specimens of faeces examined	64
Number of specimens of faeces positive	13
Excretion coefficient	59
Number of colonies from faeces tested by agglutination	47
Number of colonies reacting as { Type	4
Group	1
Mixed	42
Number of mice which died	2
Number of mice positive on post-mortem examination	2
Number of colonies from these mice tested by agglutination	14
Number of colonies reacting as { Type	2
Group	0
Mixed	12
Number of survivors killed on 42nd day	3
Number of survivors with positive spleen cultures	2
Number of colonies from these cultures tested by agglutination	20
Number of colonies reacting as { Type	12
Group	0
Mixed	8

Exps. *P* and *W* taken together afford an excellent illustration of failure to establish pure type bacilli in the faeces in spite of repeated feeding. In Exp. *P* the distribution of the serological varieties in the tissues differs from that in the faeces.

We may now consider the whole series of experiments, first from the point of view of the association between group antigen and faecal excretion, and then from the point of view of dosage.

Table I gives the more important figures for all experiments, omitting the

results of agglutination tests on the strains isolated from the faeces or from the tissues.

Table II gives the relevant details as regards the relation between group antigen and excretion, so far as the smooth strains are concerned. The first four experiments (*A-D*) are excluded because, as mentioned above, we were not at that time using an absorbed aertrycke serum, so that we could not differentiate the pure group strains from the mixed strains. The experiments in which repeated feedings were given are included with those in which the mice were fed on one occasion only, since no question of dosage is here involved. For the purpose of this analysis, the experiments in which smooth

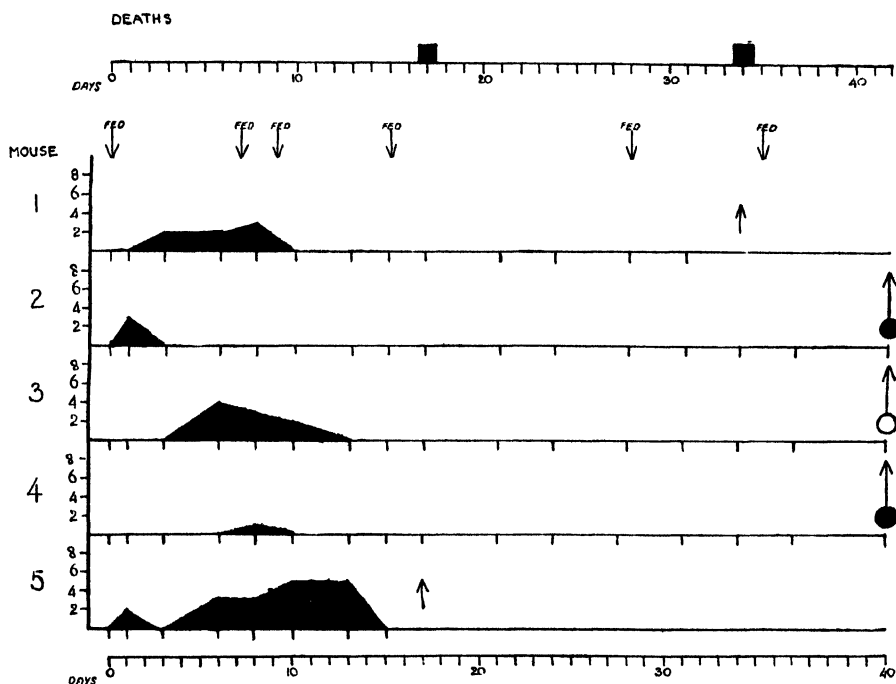


Chart VIII. Exp. P.

strains were employed are divided into three groups. Group 1 includes Exps. *E*, *F*, *L* and *W*, in which attempts were made to feed pure type cultures, and in which neither group nor mixed strains appeared in the faeces. The second group includes Exps. *G*, *H*, *M* and *Q*, in which attempts were made to feed pure group strains, and in which group or mixed strains appeared in the faeces. The third group includes Exps. *J*, *S*, *T* and *P*. In the first three of these experiments mixtures of type and group strains were fed to the mice. In Exp. *P* a supposedly type strain was administered, but mixed strains appeared in the faeces.

It will be seen from the table that the association of group antigen with faecal excretion is amply confirmed, whether we consider the results of feeding

Table I

Experiment	No. of mice	Strains fed	No. of times fed	Days observed	No. of mice excreting	Percent- age of mice ex- creting	No. of specimens examined	No. of specimens positive	Percentage of specimens positive	Excretion coefficient	Total deaths	Specific deaths	Total deaths per cent.	Specific deaths per cent.	No. of survivors examined	Spleen cultures	
<i>N</i>	10	S.T.	1	42	0	0	139	0	0	0	2	1	20	10	8	2	+
<i>F</i>	10	S.T.	1	42	3	30	144	5	3.47	13	2	2	20	20	8	0	6
<i>L</i>	10	S.T.	1	42	0	0	125	0	0	0	4	1	40	10	6	2	8
<i>B</i>	10	S.T.	1	42	3	30	110	3	2.73	11	6	6	60	60	4	2	4
<i>C</i>	10	S.T.	1	42	5	50	171	8	4.68	16	2	2	20	20	8	2	2
<i>D</i>	10	S.G.	1	42	8	80	138	31	22.48	74	4	3	40	30	6	3	6
<i>G</i>	10	S.G.	1	42	6	60	134	15	11.19	38	3	3	30	30	7	2	5
<i>H</i>	10	S.G.	1	42	7	70	98	23	23.47	76	8	4	80	40	2	1	1
<i>M</i>	10	S.G.	1	42	2	20	131	2	1.53	2	2	2	20	20	8	1	7
<i>A</i>	10	S.M.	1	42	6	60	143	56	39.16	129	3	2	30	20	3*	0	3
<i>J</i>	10	S.M.	1	42	3	30	137	19	13.87	49	2	2	20	20	8	3	5
<i>S</i>	10	S.M.	1	42	5	50	113	23	20.35	78	4	4	40	40	6	4	2
<i>K</i>	10	R.M.	1	42	1	10	132	1	0.76	2	3	3	30	30	7	1	1
<i>N</i>	10	R.M.	1	42	4	40	151	12	7.95	19	1	1	10	10	9	1	8
<i>O</i>	10	R.M.	1	42	0	0	101	0	0	0	4	0	40	0	6	1	5
<i>R</i>	10	R.M.	1	42	2	20	124	14	11.29	33	2	1	20	10	8	3	5
<i>W</i>	5	S.T.	5	42	1	20	50	1	2	8	3	3	60	60	2	1	1
<i>Q</i>	5	S.G.	6	42	5	100	75	30	40	155	0	0	0	0	4†	1	3
<i>T</i>	5	S.M.	6	42	5	100	75	14	18.67	45	0	0	0	0	5	3	2
<i>P</i>	5	S.M.	6	42	5	100	64	13	20.31	59	2	2	40	40	3	3	1
<i>U</i>	5	R.M.	7	42	2	40	75	8	10.67	32	0	0	0	0	4†	3	1
<i>V</i>	5	R.G.	6	42	5	100	80	10	12.5	30	0	0	0	0	5	1	4
<i>X</i>	5	R.M.	5	42	2	40	64	2	3.13	13	1	1	20	20	3†	2	1
<i>Y</i>	5	R.G.	6	42	0	0	85	0	0	0	0	0	0	0	5	3	2

S.T. = Smooth Type. S.G. = Smooth Group. S.M. = Smooth Mixed. R.G. = Rough Group. R.M. = Rough Mixed.

* = Two mice kept alive after 42nd day.

† = One mouse kept alive after 42nd day.

‡ = One mouse accidentally killed.

Table II

Strain administered	Number of mice excreting	Number of mice excreting	Percent- age of mice excreting	Number of specimens examined	Number of specimens positive	Percentage of specimens positive	Number of colonies agglutinating as			Number of colonies from tissues agglutinating as		
							T.	G.	M.	T.	G.	M.
Smooth Type E, F, L, W	35	4	11.4	458	6	1.3	{ 18 100 %	0	0	{ 107 67.29 %	2	50 31.45 %
Smooth Group G, H, M, Q	35	20	57.1	438	70	16	{ 4 1.39 %	150 52.26 %	133 46.34 %	{ 2 1.16 %	102 59.3 %	68 39.53 %
Smooth Type + Group J, S, P, T	30	18	60	399	69	17.7	{ 13 5 %	92 35.38 %	155 59.62 %	{ 60 35.71 %	32 19.05 %	76 45.24 %

T. = type. G. = group. M. = mixed.

Table III

Strain administered	Number of mice excreting	Number of mice excreting	Percent- age of mice excreting	Number of specimens examined	Number of specimens positive	Percentage of specimens positive	Number of colonies agglutinating as			Number of colonies from tissues agglutinating as		
							T.	G.	M.	T.	G.	M.
All rough strains K, N, O, R, U, V, X, Y	60	16	28	812	47	5.8	{ 19 10.92 %	10 5.75 %	145 83.33 %	{ 69 41.32 %	18 10.78 %	80 47.90 %

T. = type. G. = group. M. = mixed.

known type or group strains, or whether we compare the number of type, group and mixed strains isolated from the faeces in each of the three series of experiments.

It will be noted also that the ratio, between the percentage of mice excreting *B. aertrycke* in the faeces and the percentage yielding positive tissue cultures, is not constant for the three groups. Whereas 11.4 per cent. of the mice fed on type strains excreted *B. aertrycke* in their faeces, against 57.1 per cent. of those fed on group strains and 60 per cent. of those fed on mixed strains, it will be seen that 34.3 per cent. of those fed on type strains, 40 per cent. of those fed on group strains, and 66.7 per cent. of those fed on mixed strains harboured *B. aertrycke* in their tissues, including those that were killed on the 42nd day and gave positive spleen cultures.

The distribution of the type, group and mixed strains in the faeces differs from that in the tissues, where mice have been fed on mixed cultures. In Exps. J, S, P and T, of the strains isolated from the faeces only 5 per cent. were of the type variety. Of those isolated from the tissues 35.7 per cent. were pure type strains.

There seems to be ample evidence that, under the conditions here existing, the type variety can exist and multiply in the tissues far better than in the intestinal canal. Adopting the terminology suggested by one of us (Topley, 1923) in a previous report we might say that group strains or mixed strains of *B. aertrycke* are intragliscent and supragliscent, while type strains are intragliscent but not supragliscent.

Table III shows the results with rough strains, and may be dealt with very briefly. All figures for excretion are lower than with the smooth strains. We were not successful in feeding a pure type strain of the rough variety, although we had no difficulty in isolating strains which appeared to be purely of type character when tested by agglutination. The figures for the relative number of the three kinds of strain isolated from the faeces confirm those obtained with smooth strains, as regards the rarity of pure type strains, but mixed strains were far more numerous than group strains.

We may conclude this part of our report by giving the total figures for the distribution of the three serological varieties of *B. aertrycke* in the faeces. These are as follows:

Number of specimens of faeces examined	2660
Specimens positive	292
Number of strains tested by agglutination	1228
Number of these which reacted as	{ Type 89 Group 248 Mixed 891		

Thus, 1139 of the 1228 strains, or 92.76 per cent. of all strains isolated from the faeces, possessed group antigen.

Table IV

Nature of bacterial strains	Dose in c.c.	Number of mice receiving this dose	Number of specimens of faeces examined	Number of specimens positive	Percentage of specimens positive	Excretion coefficient	Total deaths	Enteric deaths	Total mortality per cent.	Specific mortality per cent.	Number killed on 42nd day	Number of these with positive spleen cultures	Percentage of positive spleen cultures
All strains.	0.1	32	308	78	25.3	86	19	16	59.4	50	11*	8	72.7
Exps. A, B, C, D, E,	0.01	32	430	58	13	36	9	8	28.1	25	22†	7	31.8
F, G, H, J, K, L, M,	0.001	32	458	50	10.9	40.6	9	5	28.1	15.6	22†	6	27.3
N, O, R, S	0.0001	32	458	12	2.6	9	6	2	18.8	6.3	26	6	23.1
	0.00001	32	437	14	3.2	11.7	9	6	28.1	18.8	23	1	4.4
Smooth Type.	0.1	10	94	8	8.5	34	7	6	70	60			
Exps. E, F, L, B, C	0.01	10	140	3	2.1	5	3	3	30	30			
	0.001	10	156	2	1.3	5.1	2	1	20	10			
	0.0001	10	149	2	1.3	4.7	2	1	20	10			
	0.00001	10	150	1	0.7	3.3	2	1	20	10			
Smooth Group or mixed.	0.1	14	142	53	37.3	133.1	8	8	57.1	57.1			
Exps. A, D, G, H,	0.01	14	191	50	26.2	72.2	4	4	28.6	28.6			
J, M, S	0.001	14	197	44	22.3	82.2	5	3	35.7	21.4			
	0.0001	14	196	9	4.6	16.3	3	1	21.4	7.1			
	0.00001	14	168	13	7.7	27.4	6	4	42.9	28.6			
Rough Group or mixed.	0.1	8	72	17	23.6	61.1	4	2	50	25			
Exps. K, N, O, R	0.01	8	99	5	5.1	10.1	2	1	25	12.5			
	0.001	8	105	4	3.8	15.2	2	1	25	12.5			
	0.0001	8	113	1	0.9	1.8	1	0	12.5	0			
	0.00001	8	119	0	0	0	1	1	12.5	12.5			

* = 2 mice kept alive after 42nd day.

† = 1 mouse kept alive after 42nd day.

THE QUESTION OF DOSAGE.

In Table IV are set out the relevant facts as regards the effect of variation in dosage on the events which we have studied. In this analysis we have omitted those experiments in which the mice were fed on more than one occasion, but we have included the four experiments in which an unabsorbed aertrycke serum was used for the agglutination tests, since the inability to distinguish group from mixed strains does not affect the results from the present point of view.

We have given the results for all strains taken together and have then tabulated separately the figures for those experiments in which we employed (a) smooth type strains, (b) smooth group or mixed strains and (c) rough strains. The observations as regards positive spleen cultures in surviving mice were not sufficiently numerous to allow of their separation into the smaller groups.

In Chart IX we have plotted curves, showing the fall in the excretion coefficient with falling dosage (a) for smooth group or mixed strains, and (b) for smooth type strains. The latter curve has little real significance except that it demonstrates again that such strains are rarely excreted. It will be

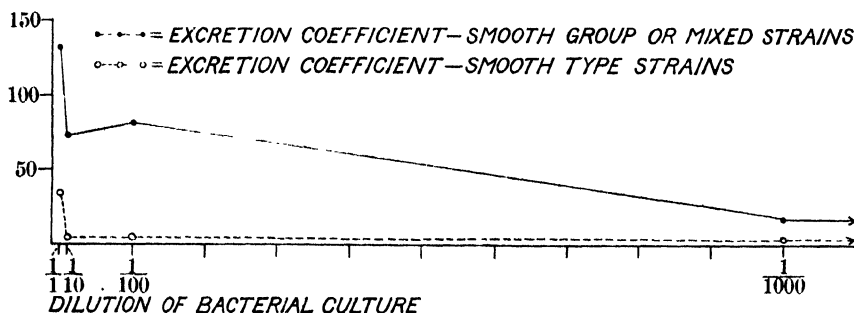


Chart IX

noted that, after the sharp fall when the dose is decreased ten times, the curve of excretion runs parallel to the base-line at a uniformly low level, indicating that it is only with relatively very large doses that any appreciable excretion occurs. The curve for the group and mixed strains is of some interest. It shows the same sharp fall when the dose is decreased ten times, but it remains at a relatively high level. It rises between the points corresponding to a ten-fold and hundred-fold diminution of the dose, and then falls very gradually towards the base-line, so that the excretion coefficient has been reduced to about 1/8th of its original value, when the dose has been decreased 1000 times. When the dose has further decreased to 1/10,000th of its original value, a point beyond the limits of the chart, the recorded value of the excretion coefficient actually rises again to about 1/5th of the figure for the undiluted culture. The total number of observations is of course too small to give any significance to small fluctuations, but we are probably safe in regarding our results as showing that the amount of bacterial culture, which we chanced

to adopt as our maximum dose, lay near to a point in the series of possible doses at which the influence of relatively small variations was rapidly changing in value; so that, while small decreases in the dose administered produced a marked reduction in the frequency with which the ingested bacilli became established in the intestinal tract of the host, further decreases, within wide limits, produced relatively little effect.

In Chart X we have plotted curves, for all strains, showing the relation between (a) decrease in dose and decrease in specific mortality, and (b) decrease in dose and decrease in the persistence of *B. aertrycke* in the tissues as judged by the percentage of positive spleen cultures 42 days after feeding. We have made an adjustment in the recorded figures in plotting the former curve. The specific mortality actually fell to 6.25 per cent. when the dose was decreased 1000 times, but it rose to 18.75 per cent. with a ten thousandfold reduction. No material significance attaches to this anomaly; the range of

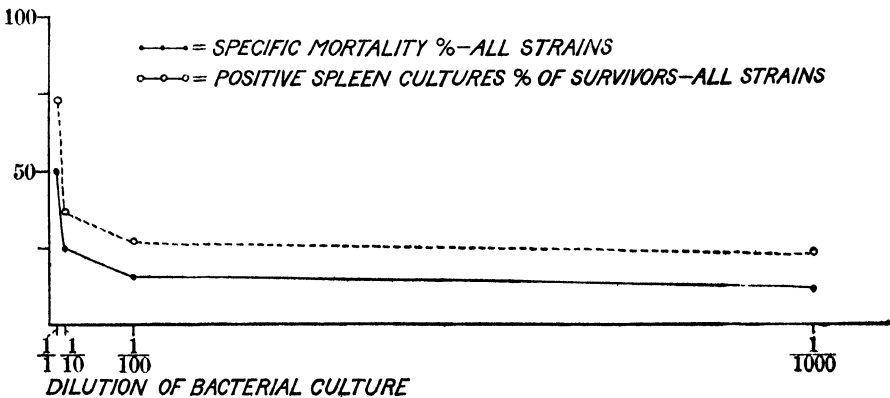


Chart X

probable variation in such small samples is large. It is impossible to record the value for the 1/10,000 dilution on the chart, and we have recorded the mean of the figures for the 1/1000 and 1/10,000 dilutions at the point corresponding to the former dose.

The points already referred to in connection with Chart IX are still more evident in Chart X, and we are inclined to think that the conclusions to be drawn from them are of some importance, and may help to explain certain differences between the results recorded by Webster (1923 *a, b, c, d* and *e*) and those which we have ourselves observed. Webster's usual practice has been to introduce 0.5 c.c. of a 1/100 dilution of an 18 hours' broth culture directly into the stomach through a silver catheter. His actual dosage thus corresponds to 0.005 c.c., a dose lying just below our second dilution, which corresponds to 0.01 c.c. There is, of course, the additional factor that Webster's dose passes directly into the stomach in a relatively large bulk of fluid, while the bacilli introduced by the method we have employed must first contend with the conditions in the buccal cavity. It seems to us very probable that

Webster has employed a dose, which, under the conditions imposed by his technique, approaches the limit at which the maximal effect of a single dose is obtained. This view is greatly strengthened by an experiment he records (Webster, 1923 *a*) in which varying dilutions of a broth culture were employed. Webster himself, in describing this experiment, speaks of the standard dose as "the massive lethal dose."

This is the dose which Webster has employed throughout, when testing different strains with regard to their capacity for giving rise to a fatal infection in mice; and he considers that a relatively constant percentage mortality among different groups of mice so treated is evidence that the strains of

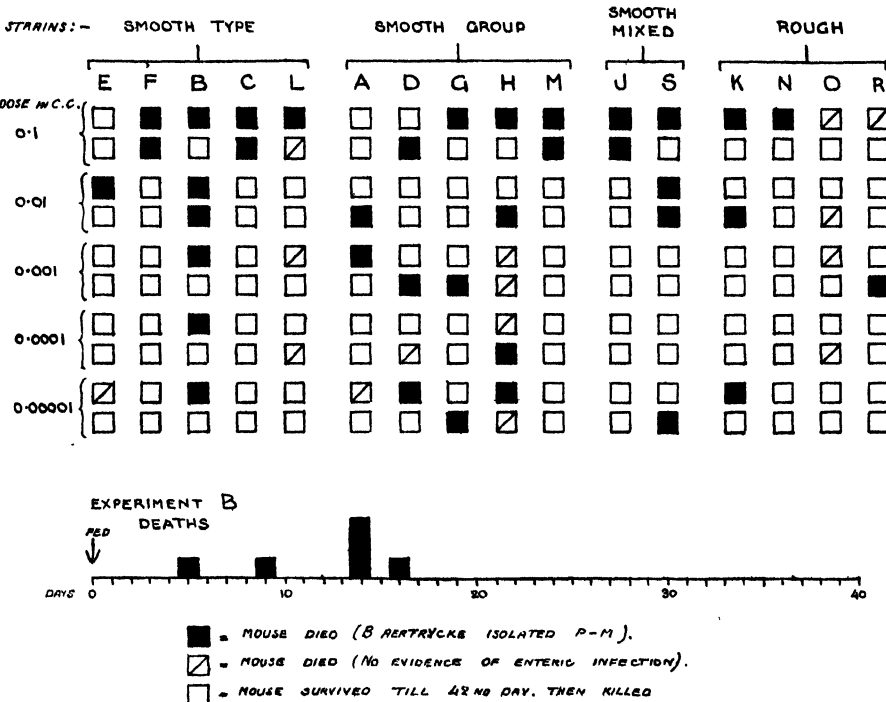


Fig. 1

B. aertrycke employed were constant in pathogenicity. It appears to us that this argument is open to objection. The observation of wide differences in the percentage mortality produced, when different strains of *B. aertrycke* are fed to mice under relatively constant conditions, may afford presumptive evidence that the strains vary in their power of producing a fatal infection. It by no means follows that the observation of a constant percentage mortality affords evidence of the absence of such variation. This would only be true if all strains examined produced like effects when they were tested over a wide range of doses. In the absence of the employment of a graded series of doses in testing each strain, the demonstration of differences in pathogenicity, if such

exist, will depend on the selection of a dose which falls within the limits between which such differences are effective.

In Fig. 1 we show the distribution of deaths in relation to dosage in each of the 16 experiments available for such an analysis. These experiments were not planned to show variations in the death-producing powers of the strain employed, and they do not form suitable material for such a study, but we have little doubt that the strain used in Exp. *B* was actually more potent in this respect than the strains used in Exps. *F*, *L*, *J* and *M*. The mortality chart of Exp. *B*, reproduced at the foot of the figure, appears to us convincing, if we consider the time-distribution of the deaths and recollect that each mouse was kept in a separate cage so that no cross-infection could occur. Yet, did we only know the results of the maximum dose, we should not assign any pre-eminent position to this strain in the scale of pathogenicity.

Although this possible masking of differences in pathogenicity between different strains, by the employment of large doses of culture, may in part explain the results which Webster has obtained, we do not believe that it is the whole explanation. It seems probable to us that the effective dose, when the bacilli gain entrance to the buccal cavity is likely to differ widely from the effective dose as measured by injection into the stomach, and that quite other factors which may have a decisive effect in determining the issue under the former conditions, may be inoperative under the latter.

DISCUSSION.

It would be altogether premature, at this stage, to attempt any interpretation of the phenomena observed in studying epidemics among mice, by applying to them the conclusions arrived at in the present report. We may, however, enquire in how far we are justified in transferring these conclusions to the natural spread of enteric infection.

It seems probable that the almost complete absence of faecal excretion, following the oral administration of type strains of *B. aertrycke*, must possess some epidemiological significance; since it affords an example of selective localisation, which, we may reasonably suppose, will have some effect on the chance of spread of this particular variety of the parasite.

Our results as regards dosage would suggest that the size of any single dose ingested, using "size" in the sense of relative numbers of viable *B. aertrycke*, will not usually be a decisive factor in determining the course of events. We cannot, however, predict that the relations, which we have shown to exist between dosage and the events which we have studied, using pure cultures of *B. aertrycke*, will hold true when we deal with faecal material containing *B. aertrycke* as one constituent of an extensive microbial flora. Until data on this point are available, we must suspend judgment. The assumption that the infectivity of a given specimen of faeces could, in any case, be predicted from its relative content in viable *B. aertrycke*, as demonstrated by any method at our disposal, is quite unjustified in the absence of

experimental proof. This question will be discussed more fully in a subsequent report.

Although we should perhaps limit our conception of dosage, in the strict sense, to the number of viable *B. aertrycke* ingested on a single occasion; yet, as we have pointed out elsewhere, the idea of dosage may be expanded to cover many other factors. The distribution of *B. aertrycke* among the mice forming the population at risk, and the percentage of these mice which are excreting this organism in their faeces, must largely determine the probability of any single mouse ingesting *B. aertrycke* with its food; and we may reasonably expect that the distribution of *B. aertrycke* in the total bulk of excrement will afford a measure of dosage, not in terms of the numbers of bacilli ingested, but of exposure to risk of infection.

It may not be out of place to emphasise certain negative conclusions.

These experiments yield no evidence that the type, group or mixed varieties of smooth strains of *B. aertrycke* differ from one another in their power of producing infection and death. The difference between them is confined to their behaviour as regards excretion. There is some evidence that rough strains produce infection and death less readily than smooth strains; but we have, as yet, no evidence that rough strains play a part in epidemics of enteric infection.

CONCLUSIONS.

(1) Different strains of *B. aertrycke* show discontinuous variation in their behaviour as regards their excretion in the faeces of mice, following administration by the mouth. These variations are correlated with variations in antigenic character as judged by agglutination.

(2) Strains which contain group antigen, alone or in association with type antigen, tend to be excreted frequently and persistently.

(3) Strains which contain type antigen alone are rarely excreted in the faeces, and if excretion occurs it is transient.

(4) Mice may suffer from typical and fatal infection with type strains of *B. aertrycke* without ever excreting these organisms in their faeces in detectable numbers.

(5) Both type and group strains may persist in the tissues of mice which have ingested them for at least six weeks, without producing any apparent change in the animals' condition.

(6) As regards the production of a fatal infection, or persistence in the tissues as judged by the results of spleen cultures, there is no detectable difference between the two varieties.

(7) Rough strains of *B. aertrycke* are less frequently excreted in the faeces than are smooth strains. When, however, excretion occurs the great majority of the strains excreted contain group antigen. Mixed strains are far more numerous than pure group strains.

(8) The mortality produced by the administration of rough strains is less than that produced by the administration of smooth strains.

(9) Variation in dosage, over the range observed in these experiments, produces its greatest effect in the neighbourhood of the maximum dose employed. Relatively small decreases in these maximum doses cause a marked diminution in the effects resulting from them. Further reductions produce relatively quite small changes in the effects observed. This holds true whether we relate dosage to the subsequent excretion in the faeces, to the percentage mortality among the mice fed, or to the presence of the bacilli in the tissues of apparently healthy survivors.

We should wish to express our thanks to Dr Major Greenwood, for giving us the benefit of his opinion on certain points in connection with this report.

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THE LIFE AND SCIENTIFIC WORK OF ARTHUR WILLIAM BACOT

By MAJOR GREENWOOD.

WITH A CHAPTER By J. A. ARKWRIGHT

(With Portrait, Plate II.)

CONTENTS.

	PAGE
I. Birth, Early Life, Some Personal Characteristics	265
II. Researches on Lepidoptera	268
III. Bacot's Initiation to Medical Research: Fleas and Plague	271
IV. Service Abroad: <i>Stegomyia fasciata</i>	281
V. War Work: The Bionomics of the Louse	285
VI. Trench Fever and Typhus (by J. A. Arkwright)	288
VII. The last Journey	299
VIII. Bibliography: A. W. Bacot's Scientific Contributions	300

I. BIRTH, EARLY LIFE, SOME PERSONAL CHARACTERISTICS.

ARTHUR WILLIAM BACOT was born in London on April 28th, 1866, the third son and fourth child of Edmund Alexander and Harriet Bacot.

The family is of Huguenot descent in the paternal line and an account of some of the members will be found in Samuel Smiles' book *Huguenots in England and Ireland* (p. 431). In physique, Arthur Bacot showed traces of his descent. He told me that on his first continental trip his companion would address a passer-by in voluble but anglicised French, while he himself, not having the gift of tongues, stood silent. Sooner or later there would come a conversational deadlock, the Frenchman would look round in despair and, catching sight of Bacot, his face would light up, he would begin a fluent discourse only to be plunged into deeper gloom by the discovery that this too was an Englishman.

Several of Bacot's ascendants and collaterals were members of the Medical profession; his great-uncle, John Bacot, served with distinction in the Peninsular War, and was the author of a treatise on the Venereal Diseases published in 1829. An older member of that generation, Frederick Bacot, a young Army Surgeon, fell at Seringa-Patam.

John Bacot's work on Syphilis—the title is a "Treatise on Syphilis," but it deals with the other venereal diseases too—is a favourable specimen of the more competently written manuals of a hundred years ago but is of no biographical interest; John Bacot's son, J. T. W. Bacot, also an army surgeon, published in 1869 a little volume on the Bahamas. This book does contain traces of a literary quality which Arthur Bacot's friends found in his conversation. "It was once rumoured in profane circles," says Surgeon Major Bacot, "that penitent pirates achieved even a higher degree of respectability in the old country,

266 *The Life and Scientific Work of Arthur William Bacot*

than the fighting chaplain. A story is told of a weather-beaten ruffian, who, after long absence from England, inquiring after old chums, was thunderstruck by the information that one of them, Blackburne, had become Archbishop of York." "Read," again says the Surgeon-Major, "the bright verses—

‘Oh, who can tell, save he whose heart hath tried,
And danced in triumph o’er the waters wide,
The exulting sense—the pulse’s maddening play,
That thrills the wanderer of that trackless way?
That for itself can woo the approaching fight,
And turn what some deem danger to delight’;

and then turn to wretched Blackbeard’s Journal. ‘Rogues a plotting! took a prize with a great deal of liquor aboard; so kept the company hot, damned hot; then all things went well again.’”

There is a characteristic Bacotian flavour in the antithesis.

As a child, Arthur Bacot was delicate and often ailing, although he was proficient at most outdoor sports, an excellent swimmer and skater (as a man he was a first-rate skater and passed the speed tests of the National Skating Association) by his eleventh year. His delicacy, called at the time “Anaemia of the Brain” was perhaps what we should now call psychoneurotic. All the children were, as Miss Bacot tells me, introspective and sympathetic to animals, a tendency encouraged and shared in by their father. Whatever we may think of its aetiology, the psychological condition led to great irregularity of school attendance and had important consequences in after life.

Psychologists tell us that the experiences of childhood are far more potent in determining what we shall become than anything which happens afterwards and certainly many of Bacot’s characteristics, both trivial and important, can be very plausibly related to his childish experiences. To begin with a characteristic, which, trivial as it was, impressed all his associates, he retained into manhood most of the likes and dislikes of the average delicate child in eating and drinking. He enjoyed at the age of 50 what a boy of 5 enjoys, and it was a melancholy thing to sit next to him at a “grown-up” dinner of many courses. He was also very irritating to folk who dieted themselves on principle. To such, a man who wore no hat and lunched off bread and jam, cheese and chocolates, seemed a long lost brother, and they were very cross when they found he was not a vegetarian, in fact enjoyed roast chicken, and had no conscientious scruples against keeping a hat in his city office (he did it, he told me, because bank cashiers and city shopkeepers with tills grew nervous if a hatless man came in). They failed to perceive that he did or abstained from doing trivial things not on principle but because he had retained the tastes of a boy.

This was a trifle, more significant were two consequences.

Being often away from school and therefore thrown on his own resources, the introspection of a child, the faculty of weaving romances out of common things, was strengthened by practice and was perhaps the basis of Bacot’s powers of sympathetic imagination. His attitude towards birds and beasts

retained something childish—in the good sense—to the end. He never canted, he did not call them his “little brothers,” neither did he adopt the intellectual manner of the schoolmaster abroad or treat fleas as the nicely educated young man is apt to treat a boy’s brigade.

The other consequence was that Bacot had no hope of escaping from his economic environment by the usual methods. The system of education of the less prosperous middle classes even more recently than 45 years ago was bad and has been dealt with faithfully by Mr H. G. Wells. The school to which Bacot was sent was very much above the level of Kipps’ academy, in fact it appears to have been an exceedingly good school of its kind and turned out several pupils beside Bacot who have made their marks in different careers, including one of the most successful novelists of our generation. But Bacot was too irregular in attendance to get the best out of the school. The methods of imparting the rudiments of mathematics, English composition and foreign languages were no doubt imperfect (they are still) but much less able men than Bacot at far worse schools than his have acquired a mastery of these rudiments. Bacot did not. He left school with no knowledge of foreign languages, and without such knowledge of the orthodox branches of science as might have enabled him to compete with a hope of success for grant-earning “Science and Art” certificates or *a fortiori* for a scholarship at the Royal College of Science.

Bacot’s difficulty in acquiring languages was not wholly due to an irregular early education. His visual memory was innately more powerful than his auditory memory; it was always remarkable that while he received and interpreted visual stimuli without seeming to attend to them, no effort of voluntary attention enabled him to retain unfamiliar pronunciations. This was an individual peculiarity; other members of his family had at least normal powers of auditory memory; but, as his sister Miss Alice Bacot is an excellent artist, perhaps the family tendency is to visualisation.

A greater handicap of Bacot’s early life was that although he had not acquired any facility of literary expression he had learned enough of so-called literary English to put him out. To the last this hampered him. In a private debating society, or, better still, after 70 miles of hard riding, sauntering through a little country town with a bag of chocolates or fruit, he was a brilliant talker, expressing his ideas with absolute precision and never hesitating for the apt phrase, the just comparison. A pen between his fingers effectually controlled this flow. His written language was often heavy and involved; he always gave the impression of struggling with a material which was repugnant to him; in fact, writing a formal paper was a task he particularly disliked. Sometimes in a private letter, more frequently in informal papers read to a little local debating society, one comes upon turns of expression which remind one of his talk; but they are not numerous. Had he been a citizen of an old Greek state or had a James Boswell been numbered amongst his friends, his literary reputation would stand higher.

268 *The Life and Scientific Work of Arthur William Bacot*

Bacot's initiation to the study of natural history only differed from that of any boys blessed with intelligent parents and brothers in its early date. He started butterfly hunting at five and his sister recollects that at the age of six the child was saddened by his kindergarten mistress's ignorance of butterflies and caterpillars.

Bacot's delicate health kept him out of the city until he was 16 but at 16 the hour struck and for the next 27 years he was employed in a city office. When I first met him, 23 years ago, he had been in business more than 17 years but he did not even then have above a fortnight's annual holiday and seldom reached home before 6.30. He had been a member of a local Natural History Society (now the London Natural History Society) for several years but he did not join the Entomological Society of London until much later. It would not be easy to imagine external conditions less propitious for the development of scientific ability, but between 1893 and 1909 he published more than 50 separate papers and contributed largely to Tutt's monumental treatise on the British Lepidoptera. The salient characteristics of this work are examined in the next chapter.

II. RESEARCHES ON LEPIDOPTERA.

Bacot's first published contribution to natural history was a note on the variation of larvae of *Saturnia carpini*, printed in the *Entomologist's Record* of 1893 (vol. iv, p. 199). It is very short and will bear citation in full.

In June, 1892, I took a brood of young larvae of this species on a whitethorn hedge near Thundersley, in Essex. From a male and female reared from these larvae, I got a batch of ova this spring; these hatched in about twenty days. After their first moult, the larvae varied very widely, some of them being entirely green or pale yellow, without any black, some remaining (until their third moult) quite black, with the exception of a reddish or brownish stripe along the side. The latter retained a large proportion of black in their coloration until nearly full-fed, while others, exhibiting nearly every grade between these two extremes, could be picked out of the brood. It seems strange that a brood of larvae should vary so widely after their first moult, and yet be so alike (comparatively speaking) in their last stage.

The following has occurred to me as a possible explanation. The larvae until the first moult, are quite black, and they feed gregariously until the third moult. When feeding on a hedge or bush they might easily be overlooked, as the effect of a brood of small black larvae lying close together is to blot out the leaf or leaves they are feeding on, leaving an apparent opening in the hedge, such as would be obtained if one or two leaves were picked off. If, however, the whole brood retained their black colour as they grew larger, the size of the apparent opening or hole in the hedge would become noticeable, but as they vary in colour, they match very well with the bright green leaves and dark spaces between, in fact, if they cleared a patch of leaves, the larvae would themselves (to a certain extent) present the appearance of the missing foliage. No doubt, after the third moult, when they scatter, the bright forms are, as regards colour, by far the best protected.

The second paragraph of this note distinguishes it from the ordinary natural history jotting.

Bacot's first long paper was read to the City of London Entomological and

Natural History Society in 1895 and printed in the *Entomologist's Record* (vi, 173-181); it deals with the Genus *Smerinthus* and the technical problem which he desired to solve was the relation of the genus to allied genera. It is not the object of this memoir to appraise the technical value of Bacot's contributions to lepidopterology—I have not indeed sufficient knowledge to permit me to make the attempt—but to note features which throw light upon the habits of mind which were a foundation of Bacot's success in medical research. I therefore restrict myself to a quotation dealing with a quite subsidiary point. He is speaking of the larvae of *S. populi* and remarks:

The young larvae have very similar habits to those of *S. ocellatus*, but as they get older, the position in which they rest is very different; this is nearly always with the head downwards, and although the fore part of the body is raised, as in *S. ocellatus*, the head is curved inwards towards the leaf or twig; they will grasp the stalk of a leaf with their anal claspers only and hang down behind it, and it is quite remarkable how small a sallow leaf suffices to hide a full fed larva. On poplars I have frequently noticed them, when they have eaten half the leaf, so resting as to represent the eaten portion themselves, and they are then so well protected that, with any wind, it would I think be impossible to detect them. I have noticed that the larvae are much easier to find on misty mornings and before the sun is up. Probably the explanation of this is, that in bright sunshine the lights and shadows are much stronger, and consequently the slight difference in tint between the larva and the leaf is not so noticeable. The same fact holds good with *S. ocellatus*, and I think that, as a rule, the protective colouration of larvae is most perfect in sunlight or in full daylight.

Bacot returned to the subject of *Smerinthus* more than once, in particular to the morphology of the hybrids of *S. populi* and *S. ocellatus* which he reared.

Bacot contributed to the 10th volume of the *Entomologist's Record*, a complete morphological study of the British Liparid Moths; it is perhaps the most compact of his morphological studies and, in the precise and methodical description of the larval stages, is a good example of what may be called the school of Chapman. The late Dr T. A. Chapman, one of the most distinguished lepidopterists of our time, had an important influence on Bacot's scientific development; their long friendship was without a break and their scientific collaboration close. A quip in entomological circles that a certain treatise, we will call it Jones's treatise on Lepidoptera, should have had on the title page the explanatory words "By Bacot, Chapman and Prout," while unfair to Jones justly enough characterises the merits of a long continued collaboration of three friends. A paper of the same type but perhaps even more minutely accurate is that on the Life History of *Agria tau* (*Ent. Rec.* xiv, 237).

From the date of his first publication to his initiation to medical entomology, Bacot's interests were balanced between morphological and genetic research. Undoubtedly the latter appealed more to him, but he probably devoted more time to the former, thereby acquiring a technical dexterity in the manipulation of small objects and a power of exact description which were to prove of great value in his later career. He perhaps owed as much to the carrying out of researches which are now interesting only to specialists, as did the greatest of British naturalists to his training on Barnacles.

270 *The Life and Scientific Work of Arthur William Bacot*

Bacot's last important contribution to pure lepidopterology was however concerned with genetics; this was the paper by Bacot and Prout communicated to the Royal Society in 1909. Bacot's chief published work on pedigree breeding had been done on *Lasiocampa quercus* and *Triphaena comes*. In the former species he had found that two races from the same geographical region when crossed produced progeny which segregated into the parental forms but that this did not occur when the southern French variety, *meridionalis*, was crossed with the Scottish variety *callunae*. Working with *Triphaena comes* from Cluny, he had 60 per cent. non-melanic and 40 per cent. melanic in the first filial generation from a melanic (male) non-melanic (female) crossing. In the next generation all the offspring of non-melanic pairings were non-melanic, but from extracted melanic pairings of like parentage, in two broods there were 30 per cent. non-melanic and 70 per cent. melanic, in another brood 21 per cent. and 79 per cent. In the next generation melanic \times melanic and non-melanic \times non-melanic pairings bred true, but the strain was becoming weakly and died out. Prout had carried out a long series of experiments upon *Xanthorhoe ferrugata*, which showed roughly Mendelian proportions consistent with the black form being recessive to the purple.

Bacot and Prout decided to experiment further with a geometrid moth *Acidalia virgularia* which offered several advantages to the breeder—its omnivorous feeding capacity, celerity of reproduction, small size and the existence of a southern French form unlike the British variety. The southern French form is distinguished by its white or cream-coloured wing ground, the London form has the ground-colour profusely dusted with dark grey atoms. The French may therefore be termed the "light" and the London the "dark" form. A complete scheme of cross-pairings was drawn up and, in all, 5531 bred specimens in ten generations were analysed.

The conclusions reached by the authors were (1) that there was no colour dominance in the light \times dark cross and (2) that the obtaining of a comparatively uniform type by selective mating and the persistence of intermediates, while not decisively in favour of any one theory of inheritance were "harder to reconcile with Mendelism than with, for example, the Galtonian view." Whether the results are explicable in terms of the Mendelian doctrine as now extended by the researches of many years, is a technical point the discussion of which does not belong to a memoir of Bacot. Although he never lost his interest in the problems of genetics and indeed did more work on the descendants of the *Acidalia* strains (this was never published), he never afterwards had leisure to devote himself seriously to the subject. From the point of view of the biographer, the importance of this investigation is its marking of a stage in Bacot's scientific self-education.

Precisely what Bacot's rank as a lepidopterist really is, must be settled by specialists. What impressed an outsider was that he never suffered from the intellectual myopia to which the self-taught biological specialist is liable. I remember lunching with him and an indefatigable lepidopterist and, their

conversation being mainly unintelligible to one without clear ideas as to the number of legs a caterpillar has, my attention wandered. But I pulled my wits together when Bacot mentioned the name of an eminent man of science of whom I *had* heard. Bacot spoke with praise of something the eminent man had done in entomology, but his friend shook his head sadly. "I'm afraid," he said, "you could hardly call X a *sound* entomologist, why"—speaking slowly and impressively—"fifteen years ago, X really did not know that the larva of YZ moults *n* times!" Bacot received this communication with a proper gravity, but afterwards—while carefully explaining to me why, in this particular instance, the specialist's horror was not quite so comic as it seemed—he enforced the lesson of how hard it is to see the wood if one pays very great attention to the trees. If *he* had ever experienced the temptation he overcame it.

III. BACOT'S INITIATION TO MEDICAL RESEARCH. FLEAS AND PLAGUE.

The joint research with Prout was Bacot's last important contribution to pure lepidopterology; the main stream of his intellectual life was soon to be diverted.

Before the publication of the research on *Acudalia* Bacot had made the acquaintance of some prominent workers in the field of scientific medicine. He had been introduced to Prof. Leonard Hill and Prof. Bulloch and, in 1908, gave an account of his breeding experiments in the Physiological Theatre of the London Hospital Medical College, one of a course of lectures on heredity in which members of the College staff collaborated. This was the first time he addressed a medical audience.

In 1909, I was employed by the Advisory Committee for Plague Investigations to analyse statistics of plague incidence; this commission and my appointment to the staff of the Lister Institute at the end of 1909, brought me into touch with the principal workers on the subject, Lamb, Liston, Boycott, C. J. Martin, Ledingham and Rowland. With the selfishness of youth, I was apt to inflict my very crude theories and opinions respecting the aetiology of plague upon my friends, and Bacot was a willing victim. He had by this time moved to Loughton and on our Saturday and Sunday walks he endured a good deal of verbiage about plague.

The subject is really a fascinating one; so much is known and yet there are such perplexing gaps in our knowledge. Why *did* plague disappear from England at the end of the 17th century? How can we account for long periods of intermission here and in India? These are but two of a hundred questions which everyone asked, and still asks, himself. The bacteriology of the disease was better understood than that of any other epidemic malady; the brilliant work of investigators in India had proved the rat flea to be an essential link of the aetiological chain, but the way this link fitted into contiguous links was uncertain. I used to bombard Bacot with questions about the ways of fleas and he would suggest, *inter alia*, possibilities of bridging over gaps in the

272 *The Life and Scientific Work of Arthur William Bacot*

time sequence. One of the first ideas he conceived was that an infection in one stage of the insect's life-cycle might be carried over into another and he surmised that a long latent period might thus be explained. The testing of this hypothesis was the topic of his first published contribution to medical entomology.

Within a few months our conversations had a practical result; I learned that the Advisory Committee were looking for someone who could study the rat flea from other aspects than the morphological, and introduced Bacot to Prof. C. J. Martin.

The gulf between the certificated professional and the amateur is not so wide in England as elsewhere, still it exists. Obviously no committee administering public funds could have been expected to invite a man of 44 whose name was unknown outside a narrow circle to throw up his means of living and become a wholetime investigator. All that could be done was to invite Bacot to take up the study of fleas in his spare time, the Committee to bear all expenses and pay an honorarium. The terms offered by the Committee were liberal, but Bacot feared that time was lacking. However, after a little hesitation, he accepted the proposal, an R.A.M.C. reservist was sent down to Loughton and with his help—he proved to be an ideal assistant—Bacot turned a derelict stable into a laboratory, collected some apparatus and a few fleas and set to work.

The time at Bacot's disposal was from seven in the evening to eight in the morning, five days a week; five hours more on Saturdays and such part of Sundays as I could not induce him to devote to forest walks. In this time he had to conquer all the difficulties of a new technic unguided by the experience of others. As I have said, he had no aptitude for languages and even if the literature had been copious it would not have helped him—as a matter of fact there was very little literature, I believe the experiments of Rösel von Rosenhoff published in 1749 were the only ones which guided him. The difficulties of his main research were sufficiently great, but in this he had only the same *kind* of obstacle to surmount as in the breeding experiments on moths from which he had learned much. But, as a side line, he took up the tracing of an infection from one stage of life-history to another, working with the house fly and *Bacillus pyocyaneus*. The tyro in a properly equipped laboratory is familiar with the difficulties of pure culturing. In an old wooden stable the omnipresence of moulds makes the work exasperatingly difficult, particularly if one has to discover for oneself all the little manipulations which the more fortunate student has had demonstrated to him by a teacher. Bacot's note on the "Persistence of *B. pyocyaneus* in Pupae and Imagines of *Musca domestica* raised from larvae experimentally infected with the bacillus," his first printed medico-entomological work (1911), is not one of his most important papers, but it cost him a great deal of patience and time.

To return to the main subject, a study of the bionomics of fleas, planned and executed under the conditions I have described, was practically completed

by the autumn of 1911. The memoir finally printed in 1914 contains some additions made after 1911 but it is substantially a record of 18 months' work at Loughton in 1910-11.

The questions asked by the Advisory Committee were these. What are the effects upon each stage of the life-history of varying conditions of temperature and humidity? Under what conditions and in what phase of the cycle can fleas tide over periods of heat or cold or dryness which are unfavourable to reproduction?

Bacot answered each of these questions fully.

I will not try to summarise the memoir but merely note a few points which are characteristic.

What first impresses a reader familiar with experimental work is the success with which large stocks of fleas were kept alive, the infrequency with which any experiment failed through the death of the subjects. This success was due to Bacot's sympathetic observation of the natural habits of his captives or his intuition of what the habits were likely to be. For instance, boxes containing laying females or cocoons awaiting emergence were buried in sand, "the habit of the flea being to hide away in crevices, corners etc., where more equable conditions of temperature and humidity are likely to obtain than elsewhere." Or again his comment upon the fact that "the mainstay of the larval diet is the faeces of the adult fleas," is illuminating.

It may be that the necessity for this diet in case of larvae of a particular species depends upon the closeness of the association between the parent and the host fed upon. Fleas being chiefly, if not exclusively, nest or lair parasites, it is not surprising to find that the larvae should utilise as food the rich store of organic matter in suitable condition for assimilation that is afforded by the droppings of their parents. It is an interesting speculation as to how far the adult's habit of wasteful feeding is the direct outcome of selective action, making a special provision for the larval food supply....The other conditions provided by the nest or lair of the host in nature are also ideal for the flea larvae, or, to put it in other words, the larval requirements are adjusted to those that are most likely to obtain where the eggs are dropped. The necessary conditions of warmth and humidity are provided by the host's body, while the provision of bedding and careful choice of a dry situation all fit in with the needs of the larval stage of the parasite. When the host leaves its nest or lair the temperature and humidity fall together, but, so far as observation goes, a fall in temperature will only have the effect of slowing development; a low humidity, however, if prolonged, will be fatal even when accompanied by low temperature. *P. irritans* [the human flea] would appear to have diverged from the other nest-breeding fleas in respect of the sensitiveness of its larvae to external conditions; possibly the progressive civilization of its host has forced it to become more adaptable. Larvae of this species were successfully reared under circumstances that proved fatal to *C. fasciatus* [the common rat flea], and, for example, were able to feed satisfactorily on crushed rat faeces when the larvae of the latter species failed. Probably the trend of selective action has been in the direction of producing a race of *P. irritans* able to feed on any possible rubbish in out-of-the-way corners. Undisturbed breeding places in such immediate vicinity to its host as to receive any appreciable quantity of the parental faeces would become gradually rarer as cleanliness and comfort succeeded to the crowding and filth of primitive conditions (pp. 472-473).

The most valuable section of the memoir is that which deals with the

274 *The Life and Scientific Work of Arthur William Bacot*

effects of varying physical conditions on the length of the larval and cocoon stage. The general conclusion reached was that in the rat flea, *C. fasciatus*, "Extremes both of heat as well as cold produce an effect which is partly of a direct nature, and partly, perhaps, a stimulus which calls into action an inborn predisposition to prolonged rest within the cocoon under unfavourable conditions. As was found in the case of *P. irritans*, it is the *changes* in temperature which appear to be the controlling factor" (p. 539).

Bacot thought that the species contained a proportion of individuals adapted for long resting periods in the face of extreme conditions whether of heat or cold but able to respond to "warm" conditions, say a temperature of 70° F., by rapid development. The evidence upon which these conclusions were based was set out in great detail, perhaps even in too great detail. But the reader who is bored by the immense statistical tables—Table XXXVIII covers nine crown octavo pages—should remember that it is the duty of a pioneer to record all his results and that these *pièces justificatives* will be of great service to any other inquirer who decides to verify any particular result upon a larger series of experiments. It was inevitable that the number of instances in any particular experiment should often be too scanty for statistical purposes; the number requisite, depending upon the variability of the phenomenon studied, cannot be determined beforehand.

The first draft of the report on the bionomics of rat fleas was Bacot's *Habilitationsschrift* and admitted him to the circle of recognised research workers; in December 1911 he was appointed entomologist to the Lister Institute of Preventive Medicine and he began work there a few months later.

Soon after he had come to the Lister we were on our way to lunch and I asked him casually how he liked his new job. He said, "Well, you know, it doesn't seem quite real yet; it is so hard to believe that nobody will come in with a bundle of accounts that I *must* go through." The Chelsea Bridge Road is not a very romantic place, but the second which followed that remark was my golden moment in life, an authentic vision of fairy land.

The two years and six months ending in August 1914 were a time of perfect happiness for Bacot. The Lister Institute was an ideal home for him. Of course he would have been happy and respected in any society of intelligent human beings, but the staff of the Lister were precisely the kind of people to suit him. All of them had enjoyed a more regular scientific training than he, but they had been trained in different schools and many of them had seen much of the world so there was none of that donnishness which is apt to make the entrance of an "outsider" into the society of men all educated on the same lines a little irksome.

But the "Oxford Manner" would have had to bite very deeply into a man's soul to make him hostile to Bacot. To some of us it was almost comical to see how quickly Minchin and he became fast friends. Minchin loved Oxford as dearly as Matthew Arnold did; one seldom passed a tea-time with him without an anecdote of Merton. Minchin's love of precise language and dislike

of the often barbarous jargon in which scientific papers are written were quite as real as Charles Mercier's. I remember the tone of his voice when he alluded to an eminent biologist who had the habit of pronouncing spermatozoon in four instead of five syllables. One might have trembled for the consequences of introducing him to a man who invariably gave spermatozoon only four syllables and nearly always stressed the first syllable of bacillus. But Minchin loved natural history a great deal better than correct phraseology and knew a real naturalist when he saw one.

For the first time in his life Bacot had command of good tools, and learning how to use them was a perfect joy. I think the tool that gave him most pleasure was a Zeiss binocular dissecting microscope and, with Minchin for his instructor, his progress was rapid. In ordinary microscopy, he soon became expert. Most students will know the value of a compliment I once heard C. J. Martin pay. He said, "Bacot never sees what isn't there."

Perhaps the pleasantest moments of his working day were at tea-time. Sometimes one could draw him out to talk about the ways of birds and beasts, sometimes it was a social-economic discussion in which he would play the part of Socrates but without wounding the self esteem of the victims, an effect which no doubt determined several votes at the trial of his prototype.

Bacot's judgments of the motives of large masses of his fellow citizens, or of their political leaders, were pessimistic. It would have been a good debating point in a tea-time argument to have called his attention to the discrepancy between his own practice and his theory. For he himself had a quite uncanny power of detecting a colleague's dislike of some little task and a habit of offering to do it himself in such a way that it was not till afterwards one realised that instead of conferring a favour one was accepting a service. He detected the Forsyte instinct in most political actions but he was not a Forsyte himself.

Bacot had plenty of interests outside his laboratory, or rather the habits of mind which gave him pleasure in his laboratory found happiness everywhere. He had a very good time at Loughton. Demographically it is a London suburb, inhabited by a fair random sample of middle class people. We are not really such mercenary fools as *Punch* artists and didactic novelists make out, but we do think that the owner of a Rolls-Royce is usually better worth cultivating in a social way than the push cyclist. We feel that a bearded, hatless man who strides past our Parish Church on Sunday morning at the hour of Early Communion pushing an exceptionally noisy wheelbarrow charged with manure is abnormal. When this disturber of the Sabbath Peace also supports the most extreme "socialistic" doctrines in a local debating society, those who know us only through literature would not expect him to be popular. But the practical justification that Bacot's life provided for what we regarded as his eccentricities was accepted. A neighbour of mine said that Loughtonians fell into two classes—those who got on with Bacot and those who did not. I have not been able to identify half a dozen members of the latter

276 *The Life and Scientific Work of Arthur William Bacot*

class. Perhaps after all we are subtle enough to distinguish between selflessness and affectation.

Besides being a London suburb, Loughton enjoys other advantages. It is in Epping Forest, and there are not many more beautiful places in England than Great Monkwood. Beyond Monkwood in the Wake Valley is a pond of deep water where we are allowed to bathe. These two places are always associated in the minds of Arthur Bacot's friends with his and our happiest hours. The Sunday ritual was to bathe in the pond and after breakfast to walk out through Monkwood. One remembers that ritual of many years so well; first the coming of Bacot (he was seldom in bed after five in the summer mornings) and his dog to rouse me and my dog, the chorus of the two dogs as we set out, Bacot's comments on the road (he would sometimes dismount from his bicycle, walk back several yards and point out moths or caterpillars which I only saw at the range of a few feet when he *did* point them out), then the swimming of dogs and men, the run home downhill. After breakfast, the forest again and Bacot's Fabre-like exposition of the little adjustments of life in Nature. All this is nothing in the telling, but in memory it is beautiful; most beautiful things are fashioned of very simple stuffs.

The volume of the *Journal of Hygiene* which contained Bacot's revised memoir on the bionomics of the rat-flea also contained the paper by himself and C. J. Martin on the mechanism of infection. To this paper the frequently abused term classical may fairly be applied.

As long ago as 1897 it had been inferred by Ogata that the flea was implicated in the spread of the plague. Simond in 1898 performed experiments which indicated that rats could be infected by fleas and his work was confirmed and extended by Gautier and Raybaud in 1902-3 and Verjbitzki in 1904. The workers of the Commission for the Investigation of Plague in India (1906-7) proved that fleas were the chief and perhaps the only means of transmission but were unable to decide what was the precise mechanism. Bacot and Martin set out to ascertain whether infection could be conveyed by the act of sucking. Fleas were fed on infected mice and then allowed to bite clean rats under conditions which precluded the contamination of the rat's skin with the excreta of the flea. It soon appeared that infection could be conveyed in this manner. Thus 20 fleas undoubtedly infected (all the insects used passed plague bacilli in their faeces) were given two opportunities to feed on each of 13 rats. Nine of these rats died of plague. In the course of the experiments it was noticed that although some of the fleas sucked energetically no blood entered their stomachs. On dissecting these the fore part of the stomach was found to be obstructed by a solid growth of plague bacilli. A special series of experiments was then instituted with fleas affected in this way. In a series of four experiments with specimens of *X. cheopis* (the Indian Rat flea) every rat bitten died of plague. Of six trials with *C. fasciatus* (the common rat flea) one led to the death of the host. Then two "blocked" specimens of *X. cheopis* were allowed to bite eight rats in succession;

of these, three contracted plague; in another trial two fleas infected three out of nine rats. This latter result was reproduced when two "blocked" specimens of *C. fasciatus* were used.

It was thus clear that fleas in this condition are specially dangerous and the mechanics of the process were displayed by serial sections. Owing to the blocking of the front part of the stomach by the plug of bacilli, blood cannot find its way into the stomach. The insect in its efforts to relieve its thirst continues to suck but only succeeds in distending the oesophagus. Given the opportunity "the insects suck blood again and again, and if the pharyngeal pump ceases for a moment, some of the blood will by the elastic recoil of the oesophageal wall be driven back into the wound and carry with it plague bacilli."

It might perhaps be thought that fleas in this condition could not long survive, but actually specimens were found to survive so long as 50 days under a temperature of 10–15° C. and 23 days at 27° C. When they died they were still infected¹.

This brilliant investigation is no doubt the one by which Bacot's name will be most familiarised to the scientific public. Self evidently, the credit for its planning and execution is only in part his, while the literary form of the paper is the work of a more skilful writer.

Two other papers of this period have to be mentioned as of importance to the student of epidemiology. Bacot's "Observations on the Length of Time that fleas carrying *B. pestis* in their alimentary canals are able to survive in the absence of a host and retain the power to re-infect with plague" are of much interest. Fleas were allowed to feed on infected animals, the latter being then removed from the cages, which were stored at a mean temperature of 47° F. for different periods. At the end of various intervals healthy mice were added to the cages. In one experiment where 47 days had intervened between the removal of the original sources of infection and bringing in clean stock, one of the latter died of plague within 24 days. It was thus experimentally proved that an infected fasting flea could continue to transmit disease after an interval of more than six weeks. Whether this were the maximum, could only be determined by a very long series of experiments; actually in Bacot's series no success was scored after a longer interval.

The other paper was on the survival of bacteria in the alimentary canal of fleas during metamorphosis and is an application to the flea of the principle involved in Bacot's first printed medico-entomological paper (see p. 304). In no case was Bacot able to demonstrate the persistence in the imago of an infection acquired in the larval stage, a result in rather striking contrast with

¹ In a further note published in 1915, Bacot figured a specimen in which the obstructing mass has a passage through it. This rupture does not restore the valvular action of the fore-stomach but allows blood to flow out of the stomach as freely as it enters. He remarked that such a flea was more rather than less likely to infect its host than a completely plugged flea. Presumably too such a flea would live longer, as owing to the patency of the digestive tube fresh blood can be ingested and absorbed.

278 *The Life and Scientific Work of Arthur William Bacot*

the success won by himself, by Ledingham, by Graham-Smith and by Nicholls in contaminating adult Diptera by infection in the larval stage.

In order to get into perspective the work actually done by Bacot in connection with plague and to explain what he had in mind to do, it is necessary and, I hope, not without interest to describe in some detail an incident of English medical history which the reader may have forgotten.

The scene of the events is a triangle of South East Suffolk. The eastern apex is formed by the confluence of the rivers Stour and Orwell, which are two sides of a triangle, the main line of the Great Eastern Railway being the third side. The sides are some 10, 10 and 8 miles long, the area agricultural land with a few small villages. The large city of Ipswich is a few miles to the North West of the area and a short distance over the north eastern boundary is the town of Felixstowe and a series of largish villages.

In the year 1906-7, the headkeeper of Woolverston Park, a large estate less than a mile from the village of Freston, noticed that rats were dying in large numbers and that the corpses showed no signs of wasting such as one might expect after a prolonged illness. The year was a dry one and this was thought a sufficient explanation.

Three miles east south east of Woolverston Park is the village of Shotley; a cottage here was occupied at the beginning of December 1906 by 8 persons, two children, a man of 56 and five women. They were all alive and well on December 8th 1906, by Jan. 7th, 1907, 6 were dead and all had been attacked by what seemed to be a very contagious pneumonia.

Three years later a similar tragedy occurred within two miles of Shotley, at Trimley just across the Orwell (and therefore beyond the bounds of our triangle, but very near it). It concerned a family of 7 persons and a little girl on a visit. Of these 8 persons 5 died between Decr. 22nd. 1909 and February 4th. 1910. This household was a very poor one; father, mother, and five children occupied a two roomed cottage. It is said that the cottage was infested with fleas.

Of the victims' signs and symptoms there were preserved details which naturally enough attracted little notice at the time, but were afterwards seen to be of significance. The mother of the family, the first to sicken, was said to have had red spots on her hands and face and a swelling the size of a small hen's egg at the angle of the lower jaw. A daughter, taken ill the day after the mother died, complained of a lump at the left angle of the jaw which was very tender—she screamed if it was touched. This girl died on Janr. 5th. A younger sister who died five days later is said to have had a large swelling on the neck. The father of the family, taken ill the day before the younger girl died, had a swelling in the right groin, it yielded no pus on incision, but finally sloughed away. The man recovered. In neither of these tragedies was any suspicion aroused that there was the least connection between the deaths of the cottagers and the deaths of rats in Woolverstone Park which the head keeper attributed to drought in 1906-7. Indeed we have now no conclusive proof of a connection; but the next incident affords some grounds for presumption.

A cottage in the hamlet of Freston, within a mile of Woolverston Park, housed two adults and five children. On September 12th. 1910, a child was taken ill; it died on the 16th. The death was thought to be due to "Gastric Catarrh and Pneumonia." The mother, who nursed her daughter, was taken ill on Sept. 21st. and died on Sept. 23rd. The cause was entered as Septic Pneumonia. The father was taken ill on the day of his wife's funeral, Sept. 26th. and died three days later. Death was certified as due to "Influenza and Pneumonia." On the same day, there died a neighbour who had nursed the mother of the family through the night of Sep. 22-23. Her death was ascribed to "Influenza and Pneumonia."

In this series of cases, suspicion was aroused. The medical attendant supplied a specimen of the sputum from the second case to a trained bacteriologist who detected bacilli morpho-

logically indistinguishable from *B. pestis*. From the third patient blood and from the fourth lung material were obtained; from all these specimens agar cultures yielded a bacillus presenting the ordinary characters of *B. pestis*. It was also reported that hares and rats had been dying in the vicinity of the cottages where this outbreak occurred. Lastly, a field inquiry was made by C. J. Martin and Sidney Rowland who detected 17 plague stricken rats out of 568 examined and mapped out a series of infected points just to the north west of the triangle and one within it.

The epizootic was indeed widely spread for five infected rats were found out of 35 trapped and examined in the Labour Colony of the Woodbridge Union, ten miles north east of the triangle.

This was the state of affairs in the autumn of 1910. It was established that at that date an epizootic was wide spread, it was rendered highly probable that the four deaths at Freston were due to infection by plague, a strong presumption was created that the six deaths in Shotley 4 years before and the five deaths in Trimley 12 months before were likewise due to plague.

In the following year a careful survey of the rat population was made and on 27 farms distributed from Alderton on the north east to East Bergholt on the south west of the triangle, plague stricken animals were found. One human life fell, that of a seaman in Shotley barracks; his symptoms were much the same as those of the earlier victims; he is said to have cut his finger while skinning a rabbit caught in the neighbourhood.

Infected rodents continued to be found in the succeeding years. In 1914 of 500 examined the bacillus of plague was isolated from 8, viz 3 ferrets, one rabbit and 4 rats. No other case occurred amongst the human population until June 1918 when two neighbours resident in Erwarton ($1\frac{1}{2}$ miles south of Shotley) died of plague. No other case has yet (1922) occurred and at the last field inquiry no rodents were found diseased.

If we conclude that the epizootic began in 1906-7, when the Woolverston Park keeper first noticed rats to be dying without obvious reason, we have the record of an epizootic continuing for at least 12 years, possibly continuously reinforced and yet responsible for only 18 deaths of human beings over the whole period. I have spoken of the possibility of continuous reinforcement, because Ipswich, within a few miles of the scene of the outbreak, is an important grain centre and had received cargoes of grain from Levantine and other ports where plague was known to exist.

Some features of the story are completely explicable in terms of Bacot and Martin's work, if we merely postulate a reservoir in the form of a steady epizootic, when a small number of the fleas which bite man will become infective¹. Of these a still smaller number will succeed in reaching a human host. But (a) the long survival period of the infective flea, demonstrated by Bacot, and (b) the very considerable powers for evil of the plugged fleas, proved by Bacot and Martin, combine to render it probable that some of the few shots will hit a mark. There remain points which are not explained, which, given an opportunity, Bacot might have explained.

Why did this invasion fail or, to speak with more caution, why has it so far failed? There are sufficient reasons why a serious epizootic should not now

¹ Martin and Rowland found that half the fleas on the rats they examined were of a species not known to bite man and, in experiments, definitely averse from doing so; they also noted that the density of the flea population on the rats was very much less than in Indian experience.

280 *The Life and Scientific Work of Arthur William Bacot*

lead to consequences for mankind like those of 300 years ago, but none to account for such a modest result as we have witnessed.

It is highly probable that the substitution of the brown for the black rat has been an epidemiological advantage; but the brown rat did not reach England (apparently in or about 1728) until a generation after the extinction of plague as a reigning epidemic.

Bacot's opinion was that success depended upon an adjustment of small details, only to be ascertained by a minute study of natural conditions, by attending to the ways of beasts and insects in the field. It was planned that he should undertake such a study of East Anglia; the late Dr Bruce Low was particularly anxious that he should do this. But more urgent work had to be performed and it is idle to speculate whether he would have discovered anything. Perhaps after all these little outbreaks *were* mere accidents of re-importation, having no deeper significance than the sporadic outbreaks which have been fairly frequent in the great seaports. Still one regrets that he did not in fact have the opportunity to look into the matter at the time of the last human cases.

Bacot published three other memoirs inspired by his association with the work of the Advisory Committee on Plague. One deals with the practical value of vapours as insecticides. He concluded that naphthalene was the most generally effective agent for the destruction of fleas in all stages of their life-history. A second, written in collaboration with the late Dr W. G. Ridewood, is an exact morphological study of flea larvae. The third paper deals with the mechanism of infection with plague by bugs.

Bacot, like Verjbitzki, succeeded in infecting rodents by the bite of bugs and he gave a careful account of the mechanism. He found that a meal of septicaemic blood was fatal to newly hatched larvae, but that an adult was capable of re-infecting a mouse after 48 days' starvation. The paper is also, I think, of interest from the biographical point of view in its improvement of form; he never became an attractive writer but his more recent papers are better knit together than his earlier ones.

This work filled Bacot's time from his appointment to the Lister Institute to the summer of 1914. In August 1913 we made our last long cycling trip together, reaching Keswick by way of the Eastern counties and Wensleydale. Bacot was a strenuous rider. The programme he most enjoyed was to start after an early breakfast, ride 25 or 30 miles, stopping to inspect any village church of merit and travelling if possible by roads not infested by motors—the drivers of fast cars, game preservers and party politicians, were the only fellow citizens with whom he was not in charity—to lunch on cakes and chocolates at a village baker's; then to ride another five and twenty miles and eat bread and jam at a wayside inn; after this, twenty miles in the evening and a supper of bread and cheese sent him happy to bed. In his later years, the care of his pets sometimes led to delays. Our very last outing together was in August 1921; we had planned to ride out to the Suffolk border and

photograph an old Manor House. We started on a sultry morning and when we were some ten miles from home Bacot remembered that he had left a box of lice on my drawing room table which would surely perish unfed. So we had to turn back and it was *very* hot. When we started off later in the day, we rode, as I thought, very fast for an hour or two; I was about to say as much and to make self congratulatory remarks upon the physical vigour of sedentary researchers over forty, but Bacot anticipated me by calling my attention to the fact that it was getting dark and asking tentatively whether I should much mind pushing on a bit. I thought of my twelve years' juniority and made a determined but wholly unsuccessful attempt to keep ahead.

In the 1913 trip, there was never the least doubt who was the better rider. I recollect our crossing a toll bridge south of Selby late in the evening. The custodian was sitting with his cronies and regarded us with pitying contempt. He eyed Bacot disparagingly and said very audibly to his friends, "Its just madness that's what it is—killing themselves they are, especially the old 'un." Then he turned to me and said "I suppose you two will be riding on to Newcastle?" "No," I said, "to Selby." "Why," he said indignantly, "that's only seven miles." Our experience of the inn led Bacot to regret that we *had* only ridden the seven miles. There was a waiter and a table d'hôte dinner, but no adequate cheese and inferior jam. At the end of our journey we stayed at the Fabian Summer School near Keswick. One evening an impromptu discussion was got up and Bacot was invited to open it. He chose the subject of diplomacy and criticised modern diplomacy in a way which was not relished by those members of the society who had a more extensive acquaintance with the literature of modern history than he had. I recollect that one of them defended our modern diplomatists quite warmly, gave chapter and verse for the wars that had been prevented, and reproved the suggestion Bacot had put forth that we might be drifting towards a war then. That was in August 1913.

In the summer of 1913 and the first six months of 1914 he continued to work on fleas. Early in the summer it was decided that he should take part in the researches of the Yellow Fever Commission and study the bionomics of mosquitoes at Freetown. He sailed a few days before the declaration of war.

IV. SERVICE ABROAD—*STEGOMYIA FASCIATA*.

Bacot spent just over a year in Freetown and the results of investigations there are contained in the Report published by the Yellow Fever (West Africa) Commission. A better idea of its quality will be afforded by a detailed examination of some sections than by a précis of the whole document. I choose for this purpose his investigation of the hatching of eggs.

Bacot began with an experiment upon the eggs of two female mosquitoes (*S. fasciata*) kept with several males. The eggs were collected daily, separated into 30 batches, submerged and watched. Some batches were kept in separate dishes, others—although of course separated one from another—were immersed

282 *The Life and Scientific Work of Arthur William Bacot*

in one large dish of water. This preliminary experiment at once showed that a change in the conditions to which the eggs were exposed acted as a stimulus to hatching. Thus batch No. 20 consisted of 80 eggs laid on 28th Sept. 1914; five hatched on 1st Oct., 24 on 2nd, six on 3rd and then no more for a fortnight. The water had gradually dried and on 18th Oct. the remaining eggs were re-immersed and on the next day eight more hatched; on 21st October more water was added and within a few hours 35 more eggs hatched out.

These preliminary trials led Bacot to formulate the following series of questions.

- (1) Does the quantity of water affect the hatching rate?
- (2) Are all eggs laid capable of survival after drying; if some only, is resistance evenly distributed through the batches laid?
- (3) Is the viability of eggs diminished by storage; if so, do the conditions of storage as regards humidity affect the result?
- (4) Is a change of temperature the adequate stimulus causing resistant eggs to hatch when dried and re-immersed, when transferred to fresh water or when fresh water is added to that in which they are already immersed?
- (5) How long after impregnation can a female continue to lay fertile eggs?
- (6) How many females can a single male impregnate and what percentage of the eggs of any one female will prove sterile after impregnation by a single male?
- (7) To what extent can resistance and the tendency to defer hatching be induced by external conditions after incubation?

First Question. This, suggested by variations in the preliminary experiment, was answered by comparing the emergence rate when eggs of the same laying were exposed in large or small pans of water. The answer was not absolutely decisive. The cleanest experiment, No. 7 of the series, showed 94 per cent. hatched out of 94 eggs in the small pan, 86 per cent. of 98 in the large pan; an insignificant difference. Exp. 8 which gave 65 per cent. of successes of 200 eggs in the small pan, 80 per cent. of 200 in the large pan might seem strong evidence in favour of the large pan but a somewhat sudden fall of temperature which occurred during the experiment might have had a greater effect upon the small than upon the large pan. An interesting fact discovered in the course of this series of experiments was the great length of time which may elapse between the submersion of an egg and its hatching. In one series of experiments, the interval exceeded four months.

Second and Third Questions. The first experiment of the series (No. 9 of the Report) concerned batches of eggs laid by a single female; half of each batch were immediately immersed, the remainder stored for at least a month. Some of the batches were spoiled by the depredations of book lice, but in a clean controlled series 82 per cent. of immediately immersed eggs (244) hatched, 72 per cent. of deferred samples (242) eggs. Another experiment on eggs laid by several females gave a practical equality of hatching rates. 38 of 66 immediately exposed hatched; 42 of 78 retained in storage 39 days;

percentages of 57 and 54. A third experiment when the storage time was increased to 82 days gave a much lower proportion: 137 of 197 immediately tested hatched, but only 51 of 196 stored. A fourth experiment where the interval was a month gave a balance of 12 per cent. in favour of the immediately immersed.

In view of the fact that there was no sensible difference between the survival rates of the first sixteen batches in Exp. 9, Bacot inferred that the answer to the first part of his second question was "yes" so that the second part did not arise. The answer to the third question was that long storage did increase mortality but that owing to the reduction of numbers consequent upon ravages of book lice (*Psocidae*) a precise measurement of the depreciation could not be made.

Question 4. Bacot divided a batch of some 1300 eggs into two lots; lot *B* was stored dry for 107 days, lot *A* immediately immersed. Of the latter, about 700—it was impossible to count them quite accurately—138 hatched in a week. The remainder were divided into two portions:

A', about 420 eggs and shells, placed in a pan containing some of the water in which they had been immersed from the beginning of the experiment, were cooled in an ice chest to 74° F. Within 1½ hours 102 hatched.

A'', about 250 eggs and shells, in a pan of water were warmed in an incubator from 80 to 95° F. Only 2 hatched in the first 1½ hours. In 2½ hours—by when the temperature had again fallen to 80° F.—6 more hatched; in another hour 26 more hatched and a further 6 by the end of the day.

On the next day both *A'* and *A''* were put into the ice chest, 3 more of *A'* hatched and 15 more of *A''*. In other words, 104 of the *A'* set hatched in response to cooling, 25 per cent. of the total. Only 2 of the *A''* set hatched in response to warming but 53, about 21 per cent. responded to a fall in temperature. In both cases a fall of temperature was the effective stimulus; it seemed a matter of indifference from what point on the scale the fall began.

The residue of these batches and the whole set *B* were then treated with the results set out in the following table.

Lot	March						April														
	25	26	27	28	29	30	2	5	6	7	8	9	10*	11	12†	13	14	15	16	18‡	19
A 1 (cooled)	—	10	—	—	1	2	1	—	—	1	—	—	7	3	41	12	3	1	—	6	3
A 2 (heated)	—	1	—	—	—	2	—	—	1	2	—	—	16	4	23	3	—	—	—	1	3
B (stored dry)	109	15	16	17	6	—	—	1	1	1	2	1	32	22	3	5	3	—	2	4	5

* On 10th April, the eggs hatched within one hour of the commencement of the cooling.

† The second cooling on 12th April was slight, the fall being only 3 or 4° F., but it was long continued (it lasted five hours).

‡ The third cooling on the 18th of April was for one hour, the fall was from 85 to 74° F.

The answer to the fourth question was therefore unequivocal, that cooling was an important stimulus.

Questions 5 and 6. The experiments answering these (No. XLVI, p. 122 of the Report) do not illustrate any new point of technique, so it will suffice to note that six females in company with a single male which emerged on 25th Oct.

284 *The Life and Scientific Work of Arthur William Bacot*

and died on 1st Nov. laid 1,139 eggs of which 753 hatched. One female which began to lay on 8th Nov. laid 110 eggs of which 55 hatched. The complete record of one of these females from 30th Oct. to 3rd Dec. was 837 eggs, 680 hatched. She laid 32 eggs on 3rd Dec., 28 of which hatched. A male which lived 21 days and kept company with 21 females was responsible for the fertilisation of 584 eggs hatched from 1,264 laid.

Question 7. 385 eggs were incubated in a moist chamber for 50 hours. 192 of these immersed immediately gave 189 hatchings within 30 minutes. From the remainder two dead, and one living larvae were extracted. The balance of the 385, 193, were dried. Upon the following day 168 remained—the others had been devoured by book lice. Of the remaining eggs 86 were immersed and 72 ultimately hatched, 18 within 24 hours. The residue, 82, were immersed seven days later; 35 hatched within 24 hours, 44 hatched in all. As Bacot wrote, the evidence is “so definite and conclusive, with regard to the determining powers of drought or humidity (subsequent to incubation) either to bring the resting habit into action or to allow it to remain latent that the only point remaining to be solved would seem to be whether the effect is invariable and relates to all eggs laid or if it is occasional only, related possibly to a seasonal change in the constitution of the eggs.”

In answering this question, Bacot made another discovery of remarkable interest, that the hatching of eggs cannot normally occur in sterile water. Preliminary results are given in the Report (p. 51) but the whole matter is discussed in a subsequent joint paper by Bacot and Atkin from which I abstract the chief findings.

The general nature of the results is illustrated by the first protocol (Bacot and Atkin, p. 502) which I give in full.

First experiment. 100 c.c. of tap water was put into each of two glass pans. The water temperature during the course of this experiment was about 65–67° F. A batch of about 350 eggs laid on filter paper three weeks previously and allowed to dry after incubation, but stored in a moderately humid atmosphere, was divided into two lots, approximately half being immersed in each pan. To the water in one pan (*A*) was added 3 c.c. of sewage water (a fragment of human faeces was placed in water and allowed to incubate for 48 hours at 80° F), to the water in the other pan (*B*) nothing was added. After one hour in *A* 4 eggs had hatched, in *B* no eggs had hatched. After 4 hours in *A* 10 additional eggs had hatched in *B* no eggs had hatched. After 20 hours, in *A* 113 additional eggs had hatched, in *B* 6 eggs had hatched. After 70 hours in *A* 9 additional eggs had hatched. Total 136 eggs, in *B* 36 additional eggs, Total 42 (p. 502).

In a second experiment under like conditions horse dung was added to pan *A* and in 18 hours 148 eggs had hatched, in the control 11. Then pan *B* was contaminated and within 18 hours 159 more eggs had hatched in that pan.

More exact experiments were then performed. The eggs were sterilised externally by repeated washings in lysol and batches immersed in the presence of (1) horse dung, (2) a sterile filtrate of *Bacillus coli*; (3) 1/1000 solution of washing soda; (4) 1/1000 solution of ammonia; (5) a living culture of *B. coli*. All the eggs in (1) and (5) hatched within 68 hours, a majority of (2), few of (3) or (4).

Further experiments proved that living bacteria or living yeast cells were in general far more effective stimuli to hatching than either acidity or alkalinity in sterile media or sterilised bacterial or mycetozoal cultures. But it was found that autolysed cultures could provide an effective stimulus so that the presence of living cells although far the most effective was not an absolutely essential stimulus.

The development of larvae after incubation was studied in similar fashion; it appeared that the presence of living bacteria or yeast cells while not essential was so greatly more favourable than any other arrangement that it was probable that bacteria or yeast cells were in fact the normal food supply of developing insects.

From the merely utilitarian point of view, the results are of some importance, since they show that the ordinary methods of purification of water from bacteria will also be of service in the reduction of the mosquito population. As starting points of further research into the bio-chemistry of growth they are still more valuable and suggestive.

A very good judge, Prof. A. E. Boycott, thinks that it is by this beautiful research Bacot would wish to be remembered.

V. WAR WORK. THE BIONOMICS OF THE LOUSE.

Bacot's war work began immediately after his return from West Africa. He had been busy with lice for some months when he wrote to me (on Feb. 1st, 1916):

I am still working out the lice, it is a slow *itching* drudgery but quite interesting in its way. I must say that I wonder the troops put up with it. I should scratch on parade but perhaps the men do. I saw an officer walking up by the barracks with his hands in his breeches pockets to-day. He turned in at the gate and only took one out—evidently we are progressing and shall win some small affair shortly. To return to the lice—on the western front, it appears to me a scandal that there should be any trouble; the whole of the people in the trenches ought to have clean underclothing once a week but if the washing of the garments is beyond the powers of the army organisation they should have new ones. I understand that the cost per man per year is about £300. For another £10 one could get a lot of new underclothing.

I fancy it is nervousness of the idea rather than the expense is a difficulty. If it were a change of ammunition it would be done, and the absence of lice would make a lot of difference.

Bacot dealt with lice exactly as he had treated fleas and mosquitoes, he adapted his experimental methods to suit the subjects, he did not confine his experiments to those subjects which happened to survive some technique convenient for the experimenter. His wartime publications were numerous but mostly short practical notes. The fullest account of his work is given in a paper read by him to the Section of Epidemiology and State Medicine of the Royal Society of Medicine in 1918.

Bacot summarised the bionomic characters of lice which are of sanitary

importance in the following propositions the truth of which he established by a long series of experiments.

(1) Eggs take seven to ten days to hatch under normal conditions, in clothing that is constantly worn; if clothing be discarded and allowed to cool for a period each day, the time of hatching may be extended to five weeks.

(2) Active lice can survive without food and apart from a host as long as nine days.

(3) Young lice attain sexual maturity in from 10 to 14 days.

(4) Females begin to lay within two to four days after attaining sexual maturity.

(5) Egg production cannot take place without food or when the temperature falls below 65° F.

(6) Virgin females cannot lay fertile eggs.

(7) A single impregnation is not effective for longer than 20 days.

(8) A female may lay from 10 to 12 eggs daily.

(9) As many as 300 eggs may be laid by one female.

Bacot considered that the most suitable method of lousing clothes was, wherever practicable, by exposure to dry heat; he found that exposure to 55° C. for 30 minutes was ample for the destruction of both nits and adults. He insisted strongly on the economic extravagance of various plans which, he once told me, were apparently inspired by the technic of the witches in *Macbeth*. The same thought is expressed in the following passage which I quote as an example of the improvement in literary expression which is evident in his later writings.

"I am rather labouring this point, because it is one of the peculiarities of the destruction of insects that economy of thought and extravagance in practice should be so general. The spirit which dominates the illustrations of Mr. Heath Robinson seems also to exert considerable control over the inventors of insecticides, whose common practice it is to endeavour to raise the general efficiency level of their preparations by complexity in combination. The following, though possibly somewhat flamboyant, is a not unfair illustration of the process: 'Kummerfeld's wash is useful, and is prepared as follows; Twenty parts precipitated sulphur are incorporated in a mortar with fifty parts glycerine; two parts of camphor are separately ground with fifty of Eau de Cologne, and twenty of borax and 870 parts of distilled water are added; the whole is mixed together, and three drops of an extract of musk are added; shaking in order to prevent the sulphur from settling down; fifty parts of ether are added to the mixture.' This principle pervades a large proportion of the pre-war remedies, and some of the recent ones, and has even crept into the work of scientific importance. For instance, it was remarked by one experimenter that cyllin water when cool was not effective, but became so when heated to 60° C; yet it had already been pointed out in his own paper that dry heat at 60° C killed the nits."

Bacot devised a method of accurately testing the efficiency of insecticides and employed it in a long series of experiments. His apparatus consisted of a body belt upon the body side of which lice were imprisoned in gauze pockets at assigned distances from a pad impregnated or dusted with the insecticide. The prisoners could feed on his body and the percentage mortality when the distance of the prison from the depot of insecticide was varied gave a quanti-

tative scale of efficiency. He concluded that, for all practical purposes, every insecticide only acted by contact. For the impregnation of garments, crude carbolic emulsified in soft soap was the most efficient agent. He found that in the summer a shirt treated with a 5 per cent. solution of the emulsion, *i.e.* containing $2\frac{1}{2}$ per cent. carbolic was efficient for five days. During the winter a like efficiency was attained by impregnation with 10 per cent. of the emulsion, *i.e.* 5 per cent. carbolic. An average sized shirt having an area of some 1600 square inches would take up about 1000 c.c. of fluid and retain 500–600 after wringing. Thorough drying before use was of course essential and flannel so treated could be stored at least 15 days before use without deterioration.

The drawback of the method was its slow development of efficiency. Of quickly acting remedies, naphthalene proved the best. Being readily soluble in benzene, paraffin, or, most important of all, oil, it could be used to impregnate garments; at a concentration of 10 milligrammes per square inch—about 16 grammes to a shirt—it killed all lice present within three hours when used as a powder, but lost its efficiency within five hours; thus it should be used in conjunction with an emulsifying agent.

In war time, the authorities received a continuous supply of worthless specifics from both knaves and fools. Bacot, as honorary consulting Entomologist to the Army Medical Department, was able to prevent a good deal of waste of money and more disappointment. Some of these incidents had their comic side. One concerned a preparation which I will call Jericho powder. It seems that the War Department had come into possession—whether by purchase or benevolence—of a large supply of Jericho powder, and a sample was submitted to Bacot for examination in the routine course. He reported that it was inefficient. Then he received a sample of Jericho powder modified by the addition of a small percentage of naphthalene; this was rather better but not nearly as good as neat naphthalene. Successive samples containing more and more naphthalene were forwarded until Bacot—grudging the time involved in the testing process—made the suggestion that, in view of the heavy demands upon the Army Service Corps, the best plan would be to send naphthalene and Jericho powder to France in bulk, to retain a dump of Jericho powder at the base and to send on the naphthalene to the trenches. I think he had no more samples of modified Jericho powder to assay.

The other case may be given in his own words.

“Two trials were made with the *Kergold anti-vermin body belt* for which the suppliers made the following claims: ‘Perfect immunity from all insect pests. The Kergold is the only belt that affords instant and permanent relief from the bane of the soldier on active service. Vermin simply cannot exist when the Kergold anti-vermin body belt is worn; protects the wearer from head to foot. Total immunity from all further attacks. The medical properties of the belt last for approximately six months.’ The first trial gave results so greatly at variance with these claims that a second belt was purchased in order to avoid the risk of the first one being a ‘bad egg.’ In the first series of trials the lice were confined in gauze pockets fastened to the shirt so that the insects could feed during the course of the test.

288 *The Life and Scientific Work of Arthur William Bacot*

In all, eight pockets were placed at varying distances above and below the belt, from the neck to the thighs; during the course of a continuous twenty-four hours' trial the only lice which died were a few in one of the pockets on the upper part of the chest, apparently from pressure of the braces. In the second trial much trouble was taken to wear the belt before trial and to induce a perspiration by work in a hot room at 97° F. Four gauze pockets containing lice were suspended within an inch of the belt and most of the insects fed heartily at intervals during a twenty-four hours' test. After this period it was found that out of sixty lice nine were dead, four were feeble, while forty-seven were active. While conceding the possibility that the dead and feeble were due to the action of the belt, I incline to the view that they were overlaid during sleep.

After the belt had been continuously worn for sixty-two hours another test was carried out. After twenty-four hours there were three dead, two feeble and twenty-nine active lice in the pockets. The belt was then worn for a further period, and after five days' continuous wear it was given a last chance. After a twenty-four hours' trial, during which many of the insects fed heartily, one was dead and twenty-nine were active, including one pair 'in copula.'"

Most of Bacot's time between 1916 and 1918 was devoted to the subject of the prevention of lousiness, but before the end of the war he had begun work upon the specific aetiological rôle of the louse, in disease transmission. In the first instance his attention was specially directed to Trench Fever; from 1919 to the end he specialised on Typhus. In the following chapter, Dr Joseph Arkwright describes this stage of Bacot's career.

VI. TRENCH FEVER AND TYPHUS.

By J. A. ARKWRIGHT.

By the autumn of 1917 the seriously reduced efficiency of the British Expeditionary Force in France as the result of Trench Fever had become sufficiently recognised to bring about the appointment of a War Office Committee to investigate the cause and prevention of this incapacitating epidemic.

Since MacNee first described Trench Fever and showed it to be an infective disease in the spring of 1915, suspicion had fallen on lice as the transmitting agents. The plague of lice was still on the Armies in France in 1917 and so far no effectual means had been taken to subdue it.

At last then the needful means for dealing with the matter were attained and in addition to a well organised hospital for trench fever cases at Hampstead with a well equipped clinical department, the laboratory side of the subject was attacked more seriously and above all the need was realised of a skilled entomologist able to undertake with knowledge and experience the management of lice in captivity and under experimental conditions.

Bacot as explained in the last chapter had made himself an expert in dealing with these elusive and, in captivity, delicate insects. An immense amount of time, thought and trouble had been given by him to finding out the best methods of handling, keeping, feeding and breeding, and maintaining an adequate stock for experimental purposes of these agelong intimates of mankind, the biology of which very few had attempted to study before the war.

Bacot, undeterred by the disagreeable and irksomely frequent needs of his subjects, devoted himself to the task and, with unfailing regularity pro-

vided fresh stocks as required, carefully graded as to age and not open to doubt as to their uniformity of race and freedom from disease. These he had fed and reared on his own blood under definitely known conditions. Not only did he thus provide an absolutely essential element for the W.O. Committee research, but he gave unstinted help, advice and stocks of lice to workers in other places including the American Committee in France.

To the uninitiated it may seem that the public service of providing a supply of normal lice for research was a matter which could have been easily performed by any unskilled assistant. That was not the case, though later as the technique became surer it was possible to get much help from specially trained careful laboratory assistants. The devotion, knowledge and skill needed to have at hand for experiments a large number of healthy, young lice all of approximately the same age and carefully confined in suitable boxes, were attained to by very few to anything like the degree reached by Bacot. Many have tried and failed. In the care of the untrained in spite of advice the lice invariably died. To the ignorant or selfishly fastidious such personal devotion may not appeal.

Besides his work for the W.O. Committee, Bacot gave much help and advice to sanitary and school authorities who rightly dreaded the extensive introduction of lice into England by soldiers from the trenches. Though he was always ready to do all he could to help schemes for stemming an invasion by vermin and was not by any means blind to the unpleasant or even dangerous possibilities, Bacot expressed the opinion that so long as the economic conditions of the general population were not seriously depressed and the housewife was able to maintain the weekly wash, body lice would not be able to get a hold in the family to any serious extent.

During the investigation Bacot at first spent much time going to the New End Hospital at Hampstead where Major Byam and his staff were working at the clinical side of trench fever. He also put in long hours of work at the Lister Institute, Chelsea, making detailed dissections and examinations of lice as well as continuing his disinfection experiments with chemical substances and methods reputed to destroy lice and de-infest the men in the trenches. At the same time he usually had at least one other entomologist working in his room to whom he gave ungrudging help. Soon after the Trench Fever Committee had been formed, Capt. Peacock, the entomologist who was working at Hampstead, returned to France and Capt. Lloyd took charge of the work at the hospital, while Bacot maintained control and gave much help.

The result of Bacot's skilled entomological work was soon apparent and with his assistance exact experiments of different kinds became possible, which could not have been undertaken before. His careful system of numbered boxes of lice and his knowledge of their needs—how often, and for how long they must be fed, how best they should be applied to the skin, at what temperature and at what degree of moisture they should be kept between feeds in order

290 *The Life and Scientific Work of Arthur William Bacot*

that they might be maintained alive and active—these and many other points of detail were most valuable practical contributions to the advance of the investigation. The best methods of rapidly increasing the stock or keeping down the numbers by varying the frequency of the feeds and the temperature at which the insects were kept, without wasting time by unnecessary sorting and manipulation, had only been learnt by continuous care and observation; information as to the extent to which with proper precautions feeds could be omitted for any reason was an important factor in the success of the experiments. The energy and organisation of Major Byam and the generosity of the Lister Institute brought to use the self-sacrifice of a number of volunteers, and, by experiments with lice which had been previously carefully fed on trench fever patients, it was shown that the virus was contained in the bodies and excreta of these insects, and that it could reproduce the disease in man. Simultaneous work by the American Committee in France led to similar results. It was also established that the easiest and probably most frequent manner of transmission from man to man in the trenches was the inoculation of louse excreta into small skin lesions.

The high state of concentration in which the virus was found to exist in the excreta of lice a definite time after they had fed on a trench fever patient opened the way to the investigation of many further problems. Microscopic examination of the excreta and the lice showed that both contained the *Rickettsia* bodies characteristic of the disease and Bacot threw himself with zeal into the study of the conditions needed to ensure the appearance of these bodies in lice, and of their exact location in the insect.

The latter question he elucidated by laborious microscopic dissections and sections of the lice cut with a microtome.

This work went on till in 1919 the end of the war and the establishment of less arduous conditions in the army brought trench fever as a recognisable infective disease to an end. Among other problems tackled was the question of heredity in the louse of the virus and of the *Rickettsia*. This appeared to be settled in the negative sense. Whether larvae and nymphs could become infected and whether the *Rickettsia* appeared in them as in adults were answered by showing that the age of the lice was not a condition controlling the development of either virus or *Rickettsia*.

As may be imagined the labour which all this entailed was considerable. During this period Bacot was working early and late and can only have spent very few hours out of the 24 at Loughton. His eagerness to leave no part of his share of the work undone and indeed to think of more tasks in connection with it were a constant source of surprise and admiration to his colleagues. No amount of work that he could do nor time that he could spend in furthering the investigation seemed too much. His nights must have been short and his meals were often taken when and where he could find time to eat apples, biscuits and chocolate which he carried with him.

In December 1918 fresh problems presented themselves to him. The re-

semblance of typhus fever to trench fever in the apparently exclusive part taken by lice in the transmission of the two diseases and the reported similarity of the visible accompaniments of the virus in lice led naturally to an attempt to throw light on this disease also. The presence of typhus at that time in the north of Ireland enabled the Lister Institute workers to procure some infected lice. Monkeys and subsequently guinea-pigs were infected and feeding lice on infected animals was undertaken with enthusiasm by Bacot. His aim in these experiments as in those connected with trench fever was to obtain as accurate results as possible.

Lice were therefore used in limited numbers, about 20, in each box, so that each louse came under his supervision. The number of males, females, nymphs or larvae was recorded, and the number dying daily with the results of dissection of those found dead or killed were noted. Difficulties of detail arose for which he was on the watch and which he was often successful in overcoming. One difficulty was the reluctance of human lice to feed on a monkey, and it was soon found that they did not readily suck monkeys' blood through the gauze covering of the boxes but had to be let out to browse free on the skin. This entailed a long and vigilant watch over the feeding flock and much care lest they should stray too far and escape. The risk from escaped lice was thoroughly recognised. In spite of all his care there was always a large mortality among lice fed on monkeys.

Bacot was able to establish several interesting facts during those trying hours of feeding. He had shown before that head and body lice could equally well be infected with *Rickettsia quintana*, the species associated with trench fever, and that both were capable of transmitting this disease. He now found that *Pediculus capitis* was better suited to feeding on the hairy skin of the monkey than *P. corporis* and was therefore a much better stock with which to conduct experiments on monkeys. It was also shown that both the forms of *P. humanus* could be infected from a monkey both with typhus virus and the associated *Rickettsia* (*R. prowazeki*). One louse with these contents was a sufficient dose to infect a guinea-pig or *Macacus rhesus* when inoculated subcutaneously. In the course of the work it was found that macaques when infected and ill with typhus, often became infested with swarms of a small louse peculiar to the monkey, *Pedicinus longiceps*, though few if any of the vermin were to be found when the monkey was in health. Bacot was intensely interested in all the phases of parasitism and these occurrences afforded him much food for thought. He translated his observations on the monkey into terms of probable human experience and infestation. His reflections and deductions from the facts were often original and quaint and were suggestive of equal sympathy with the insect and the host.

The monkey lice taken from a *Macacus* ill or dead of typhus proved to be infective for a fresh monkey and also to contain *Rickettsia* very like and apparently identical with those found in *P. humanus* infected with typhus. This made a new and to Bacot fascinating line of research which was laborious

and difficult and moreover limited by time and the supply of monkeys. The *Pedicinus* did not lend itself to culture in boxes like *Pediculus humanus*, but one consolation was afforded by its refusal to bite human beings.

The typhus problem presented many puzzles and Bacot's active mind well stored with insect lore was constantly at work endeavouring to visualise the behaviour of the virus in the insect vector and the reaction of the insect to it, and speculating how far the virus was in true symbiosis with the louse and how far pathogenic to it; whether the cycle of louse to man and man to louse was likely to be continuous or interrupted, and how far the effect of different seasons on the interaction of virus and louse and louse and man was sufficient to account for lacunae in our knowledge of the epidemiology. He searched with great care and diligence for microscopic evidence of the infection of the mouth parts, salivary glands and other organs of infected lice, in order to throw light if possible on the actual method of transmission to man, but no evidence of this was forthcoming to encourage the belief in infection by the bite. The even more important question of the possibility of transmission of the virus from one generation of lice to the next through the egg appealed to him still more strongly. He examined the fluid of the body-cavity, the ovaries, eggs and unhatched embryos with great skill and patience, and he believed that he had found indications that *R. prowazeki* might infect the egg. The evidence was not sufficient to satisfy him completely, though it tended to confirm the scanty evidence of occasional transmission of virus from adults to their offspring brought forward by others.

Bringing his experience and success in rearing and keeping other insects to bear on the work, he endeavoured to infect the bed-bug (*Cimex lectularius*) with typhus and we were at first surprised and interested to observe bodies in many ways very like *R. prowazeki* in bugs fed on typhus animals. This was soon found to be a *post* and not a *propter* sequence for in all specimens of this species of *Cimex* examined the same form could be found if diligently searched for. The bugs after feeding on typhus animals were moreover not infective for guinea-pigs. This refutation by Bacot himself of the suggestion that bed-bugs were perhaps capable of harbouring the typhus virus, founded on the first observations of typhus-fed *Cimex*, brought no reaction in his mind leading to the neglect of this interesting association, as it probably would have done to his colleagues.

He immediately saw that a study of this form in bugs might be a help to the understanding of the relation of other kinds of *Rickettsia* to their insect hosts and eventually to the associated diseases of mammals. The infestation of *Cimex* proved an interesting one, for this *Rickettsia* (*R. lectularii*) was found to occur in both coccus-like and thread forms as was subsequently shown to be the case with *R. prowazeki* in lice, and it also resembled the latter parasite in that it was found in large numbers inside certain cells of the alimentary system. In the case of *R. lectularii* the cells infected are those of the malpighian tubules, whereas *R. prowazeki* inhabits the cells of the gut itself.

Bacot further pursued the subject and found that the body-cavity, organ of Berlese, ovary, eggs and embryos are often infected with the *Rickettsia lectularii* and that this latter is undoubtedly passed on from parent to offspring through the egg.

In the spring of 1920 Bacot was asked to join the United States Red Cross expedition to Poland to investigate typhus, the American entomologist who was to have gone being prevented from accompanying his colleagues. Bacot fully realised that the work and especially the handling of typhus lice would be risky and especially so to a man of his years, but he did not hesitate a moment on this account and enthusiastically began to plan the procedure best calculated from the entomological point of view to advance the knowledge of typhus, and to consider how to obtain the best results with the least danger to his colleagues and himself.

In considering the risks which he readily incurred at this time and subsequently, it must be remembered that he was not a medical man and therefore not accustomed from his youth to look upon association with disease as part of the day's work; nevertheless his contempt for a safe life at the sacrifice of progress in knowledge was complete.

The journey and time spent in Poland were full of novelties in which he took a deep interest from many points of view. The climate, vegetation, manners and customs of the people all fed his imagination and his thirst for new facts.

He found the opportunities for research rather more confined than he had hoped, but was able to do some good work dissecting and examining lice, and was filled with admiration for the work of Prof. Weigl who was at that time working at Warsaw. Though their means of communication were limited owing to the language difficulty they were able to exchange information. Weigl showed Bacot the remarkable technique which he had devised for infecting a louse with typhus virus and subsequently feeding the insect by injecting blood into its gut *per anum* through a very fine pipette. Bacot took up the method with enthusiasm, made his own minor modifications in the technique and by this means was enabled to continue his work on typhus in England. The great obstacle to work at home which this method overcame was the difficulty in finding a host on which to feed typhus-infected lice, since human lice thrive badly when fed (with considerable difficulty) on a monkey, and could not be nourished on other laboratory animals, while human immune typhus convalescents were not available in London. Bacot attained great skill at this technique and was able to give an infecting feed to lice, and thereafter give them two feeds daily, all by means of a pipette, and to keep the insects alive in some cases for over 20 days. At the end of a prolonged period of artificial feeding only a few out of the initial number of infected lice had survived; some succumbed to the intense infection with *Rickettsia*, some to bacterial infection, and a few to injuries received during the feeding operation. Even under the most favourable conditions normal lice fed in the natural

294 *The Life and Scientific Work of Arthur William Bacot*

way did not as a rule live longer in boxes than about 30 days (average 27–34, maximum 46 days).

This amazing and spectacular technique required much skill and patience, but fine manipulations were always attractive to Bacot and in them he excelled.

The work in Poland was unfortunately shortened by an attack of trench fever, which he apparently acquired whilst examining lice from persons believed to be healthy or at any rate not suffering from nor associated with typhus fever, as far as could be ascertained. Some of these lice examined between the 31st March and the 5th April had been found by Bacot to contain *Rickettsia* exactly like those found in England in lice fed on trench fever patients. On the 17th April he had a sharp attack of fever and was removed to hospital on the suspicion of typhus fever, but his temperature fell to normal in two days and then he had two or more characteristic relapses. Normal lice which he was feeding on himself before and during illness excreted no *Rickettsia* till the 27th April, when *Rickettsia* began to appear in the excreta, and thereafter lice which he fed on himself yielded microscopic *Rickettsia* on and off for nearly four months. These observations and the preparations from these lice were made by Bacot himself at the time or from material set aside then and examined as soon as he was able to return to the laboratory. Some of these films are still extant and show typical *R. quintana*. Bacot published an account of his illness in the *British Medical Journal*, 29th Jan., 1921, and the lesson to be drawn therefrom, that a disease like trench fever may exist without being recognised in a louse-infested population is pointed out in his paper. He was very much impressed by the inertia and mental depression induced in himself by this illness and he described with something like awe the disinclination for work which he felt as a sensation which to him was new and strange. This experience elicited from him renewed sympathy for the soldiers who during the war had been deprived of energy and efficiency by what often seemed a trivial illness to onlookers and consequently procured for them little consideration.

After his return to London Bacot was able by means of the new technique learnt from Weigl to enhance enormously the evidence of close association of the typhus virus with *R. prowazeki*. After the virus had been passed through a number of guinea-pigs, a monkey and then another guinea-pig in series, he injected a number of lice *per anum* with minute quantities of blood from the last guinea-pig in the series and was then able to demonstrate the presence in them of virus and *Rickettsia*. After a suitable incubation period the presence of the virus was shown by inoculating one of these lice into a guinea-pig and the *Rickettsia* were demonstrated microscopically.

In 1921 he worked with Dr Ségat on the question of the virus of typhus being especially closely associated with the platelets in the blood of man and other mammals. The attempt to render lice infective by injecting them *per anum* with a thick emulsion of platelets was very successful. Previously Bacot

had tried to infect lice in this way with the blood or brain of typhus guinea-pigs, but the results had been uncertain and only successful in a small proportion of the insects used. By the use of platelets from infective blood a sure infection was obtained and with the experience which Bacot had now acquired failure on account of bacterial infection and consequent death of the lice was a much rarer event.

After many observations he came to the conclusion that bacterial infection of the gut of a louse was usually rapidly followed by its death. Three sets of observations helped towards this conclusion:

(1) The early examinations by means of films of the contents of lice without much attempt at the segregation of the parts had given the impression that bacteria were often present in large numbers inside the normal louse, but careful dissection and examination of the separate organs led Bacot to the conclusion that few if any bacteria were ever to be found in the healthy internal organs including the alimentary canal. This was confirmed by the examination of numerous sections. An explanation of the first observations of numerous bacteria in the films was found in the existence of a constant infection of the sheath of the penis with masses of a large bacterium (*B. pediculi*) which appeared to be constant in its characters in strains derived from different lice.

(2) Lice sometimes died or were found in a very feeble condition a day or two after anal feeding by a pipette and were then almost invariably found to contain many bacteria in the gut. An improved aseptic technique much reduced this cause of death.

(3) It was the view of Friedberger, and an almost identical hypothesis was accepted by Weil and others, that the virus of typhus was identical with the *O* form of *Bacillus proteus* X 19 which had given such remarkable and uniform agglutination results with the blood of typhus patients. The injection of lice *per anum* with this bacterium appeared to be a way of testing this hypothesis, and Bacot made several attempts to produce a lasting infection of lice with this strain of *proteus*. All these experiments led to a massive infection of the gut and the early death of the insects, although very small doses of a weak bacterial emulsion were administered and the effect was tried of keeping the lice at different temperatures after the injection. The results gave no support to the suggestion of identity of *B. proteus* with the virus of typhus or *R. prowazeki*.

Bacot gave much time to the study of the morphology of *R. prowazeki*, and finding that he could obtain a certain and unusually severe and rapid infection of lice with *Rickettsia* by injecting them *per anum* with a minute quantity of an emulsion of the gut of a previously infected louse, he adopted this method of culturing *in vivo* as a source for his microscopic preparations.

The lice infected in this way had subsequently to be fed twice daily by the same fascinating but laborious technique. In the material obtained in this way he found a great variety of forms of *Rickettsia*. Of these he retained an

accurate recollection and made many drawings, hoping to be able to throw light on the nature of these parasitic microbes and endeavouring to link them up with certain forms which he had seen in blood films from human patients and typhus guinea-pigs. How far the hints and suggestive appearances which he detected in blood films are really related to phases of *Rickettsia prowazeki* under different conditions, he never obtained sufficient evidence to decide.

All this time he was framing and trying to test hypotheses of the origin and phylogeny of the different kinds of *Rickettsia* and their relations to their hosts, and was tracing analogies with other insect parasites. These speculations led him to obtain and examine other species of *Cimex*, lice from other animals than man and monkeys, and the wingless blood-sucking flies (*Melophagus* and *Stenopteryx*) parasitic on sheep and birds. Much of this work demanded considerable energy and enthusiasm in order to obtain the insects, breed, rear, feed and dissect them, in addition to his other work, and was left in a very imperfect state, but it constantly suggested fresh views of the subject to his eager mind.

In 1920-21 Bacot became especially interested in *Rickettsia melophagi* which infects the intestinal canal of the sheep ked, *Melophagus ovinus*, and is apparently "hereditary" being passed on from the parent insect to the offspring through the egg. The ked is in some respects not quite so satisfactory as a host in which to study *Rickettsia* as the louse, since in the case of the former insect there is no known mammalian condition correlated with the presence of *Rickettsia* and all adult keds appear to be affected; moreover cultures of the *Rickettsia* could not be obtained though Noller had previously reported success in this direction. Observations therefore could only be made by dissecting and examining the parts of the ked at different stages of its life. The results nevertheless were interesting and unexpected. Strong evidence was obtained that in the hind-gut of the adult insect and in the mid-gut of the newly emerged adult before it had sucked blood, forms of the *Rickettsia* occurred which were much larger and more like a wide bipolar staining bacillus than the usual minute forms. These observations interested Bacot very much because he believed that he had seen somewhat similar forms of *R. prowazeki*, but only rarely, and had been unable to identify them with certainty. He hoped that eventually the life-histories of *R. melophagi* and *R. lectularii* would throw much light, from the point of view of ontogeny and phylogeny as well as epidemiology, on *R. prowazeki* in the louse infected with typhus. His ideas on the origin and degree of parasitism of the different kinds of *Rickettsia* were continually being turned over in his mind and modified by fresh evidence. He tentatively regarded the species of *Rickettsia* as parasites originally derived from the mammalian host and adapted in different degrees to the special blood-sucking insect. *R. prowazeki* was probably a relatively recent acquisition by the louse since it damaged the gut of the host and probably often caused its death, but *R. quintana* was better established and did not harm the host since it merely inhabited the lumen of the gut. *R. melophagi* and *R. lectularii* had

become thoroughly adapted as symbionts causing no serious harm and infecting generation after generation through the egg without the need of a mammalian intermediary. These views never reached in his mind sufficient definiteness for him to express them in print, but he was always on the look out for facts for or against these hypotheses, and they serve to show the bent of his mind. The possibility that the resemblance between different kinds of *Rickettsia* was only superficial was not forgotten.

During 1921 experiments by Bacot and Atkin with the excreta of lice feeding on a typhus monkey gave results which were taken as evidence against the infectivity of such excreta. This was perhaps unfortunate since the negative results biased the experimenters against the likelihood of man being infected by similar material. It may be mentioned however that attempts to infect by the bites of infected lice were also negative and Bacot was sceptical about the direct transmission by the bite as the result of his examinations of the head-parts etc. of infected lice by dissections and sections and also because of the previous experiences of the English workers with trench fever lice.

In November 1921 the invitation came from the Egyptian Government to spend five months in Egypt working at typhus in a well-equipped laboratory in Cairo with the expectation of an abundant source of typhus virus in the fever hospital and the probability of assistance from typhus convalescents in feeding infected lice. It seemed a great opportunity to investigate doubtful points under more normal circumstances and with fewer obstacles, to examine more thoroughly the development of *R. prowazeki* in lice and to gain more exact information about the relations of lice and *Rickettsia* to the epidemic. He accepted with alacrity and the present writer who went with him as a colleague had the advantage of his skill, knowledge and comradeship.

Bacot arrived in Egypt on the 5th of February and started work next day at the laboratories of the Public Health Department full of energy and enthusiasm. Dr Charles Todd the director and all his staff gave every possible assistance, the laboratory was well appointed and all promised well. Dr Sami at the Abbassia Fever Hospital also helped in every possible way. It was strange that in the early part of 1922 the cases of typhus in Cairo were very few and instead of hundreds of cases being admitted to hospital as often happened there were barely ten. However, two cases were admitted in February and from them a supply of virus was obtained at the laboratory and work was soon in full swing.

Whilst waiting for typhus material an attempt was made to sample the pediculi from the poorer inhabitants of Cairo by obtaining lice for dissection from a number of labourers, etc.

This enquiry and the work on typhus-infected lice and laboratory animals was stopped by the illness of both the workers, but such observations and experiments as there was time to make have been recorded elsewhere (Arkwright and Bacot, *iv*, 1923). Amongst other lines of research the question was again taken up whether the excreta of typhus lice contained the virus in an

298 *The Life and Scientific Work of Arthur William Bacot*

active state and as a result there was no doubt that the excreta of lice infected by Bacot *per anum* were highly infective for guinea-pigs. It therefore seemed highly probable that the excreta of lice fed naturally on typhus patients would also prove active. Nicolle had affirmed the infectivity of the recently passed excreta of lice in 1914 and again called attention to his experiments in 1921, though Rocha-Lima and others had failed to infect guinea-pigs by the inoculation of similar material.

There seemed therefore to be need for further experiments with excreta. The handling of a number of boxes containing typhus lice in order to allow the insects to feed on an immune typhus convalescent and the collection of the excreta from them was a necessary part of the work. Moreover Bacot was not satisfied to treat a box of lice as a whole but, as often as time permitted, he removed the gauze covering from each box, counted the survivors, and removed and dissected the dead or sick lice, in order that as much as possible should be learnt of the progress and the extent of the *Rickettsia* infection in the lice. I well remember his distress at the suggestion that some work should be undertaken that he thought would hinder the finer entomological part of his research, *i.e.* careful dissection of all the lice, etc. It seems almost certain that he himself became inoculated with a minute quantity of the excreta of lice through a small scratch or abrasion of the skin or possibly through the conjunctiva or nasal mucous membrane. In spite of continual watchfulness he had been unable to detect any escape of infected lice, but the contamination with excreta of the fingers could not be altogether avoided.

On Sunday 26th March he had a headache with a rise of temperature to 99° F. and felt poorly; on the next day he maintained that he felt very well and insisted on going to the laboratory where his temperature was found to be 101·6° F. The onset of his illness was rather unusually gradual but he was decidedly ill on the 29th and went to the fever hospital on the 30th. He appeared to be quite conscious till the 4th April on which day he made a partially successful effort to read a letter. His enquiries whilst in hospital were almost entirely about the progress of work at the laboratory. His attack of typhus was of a severe type but on the 7th April the temperature fell about 2° F. and remained between 100° and 101° F. and hopes were entertained of his recovery. Nevertheless his temperature never quite reached 99° F., the lung complications became worse and he died on 12th April.

The failure to rally after a definite crisis caused intense disappointment and sorrow to his friends and to Dr Sami, the medical officer in charge of the hospital, who had spared no trouble in his attendance and whose skill and experience in dealing with typhus cases was very great.

Bacot's intensely active life was ended in the midst of work into which he had put his whole heart and mind. Entomology was his hobby; the problems connected with the study of insects and ingenious methods for solving them were his pastime. The harnessing of entomology to the use and advance of human society was to him a great achievement—the realisation of a high

ideal and the fruit of the labours of a long line of entomologists past and present. He worked as hard as it was possible to work in order to fulfil, as far as might be, his ideal of human aim.

His interests were by no means confined to entomology. He had definite ideas and convictions as to the course which should be taken for the reform of Society in several directions. He was inclined to be pessimistic in his views of the ways of the world and especially of politicians, but his pessimism was of that amiable sort which, whilst distrustful of many human agencies, almost always saw the best side of and made excuses for those human beings with whom he came into immediate contact.

Though latterly he did not find much time for reading he had a keen appetite for any knowledge of human sociology ancient or modern. For this reason as well as on account of their inherent beauty he was enthusiastic about the art, monuments and tombs of ancient Egypt for which he spared a few hours as recreation. He displayed a surprising amount of information, learnt mostly in past years, about archaeology and ancient and modern human customs and beliefs.

In this subject as in his entomological work he loved to ponder over and try to connect into a consistent whole the fragments which he saw and of which he read.

As a colleague he was always loyal and considerate and anxious to help in any way. The chief difficulty in working with him was that of trying to keep pace with his amazing activity of mind and body.

He had no jealousy in his composition and his chief anxiety in publishing accounts of his work was to give recognition to the right person, and as far as his own contributions were concerned to see that any credit due should be given to him as a member of the staff of the Lister Institute rather than as an individual.

VII. THE LAST JOURNEY.

In the preceding chapter Dr Arkwright has described the scientific work which occupied the last years of Bacot's life, there only remains to add a few words on personal aspects. Bacot suffered not less than the rest of us from the post-war reaction; he had never indeed cherished dreams of a new heaven and earth to follow victory, but his temperament was not that of the prophet who can draw comfort from the fulfilment of his forebodings. I think he suffered much and his comments upon public affairs were bitterer than before. But he was not unhappy, for he never lost the key of fairyland given him in 1911.

His visit to Poland in 1920 was not, for various reasons, including an attack of trench fever, an agreeable one; his last journey began under happier auspices. His letters from shipboard were in his earlier vein, the voyage down the Mediterranean delighted him; the laboratory and colleagues at Cairo were thoroughly congenial and the last letter I was to have from him the most cheerful of all. It was written with the knowledge that a serious cause of anxiety had been lightened. That letter was written on 24th March, 1922;

300 *The Life and Scientific Work of Arthur William Bacot*

within a few days he was taken ill; an oblivion soon followed—in his delirium he spoke only of the research; on 12th April he died. His body was carried to the grave, in the British Cemetery of Old Cairo, by his friends and colleagues from the laboratories, both British and Egyptian.

In this memoir two friends have attempted to describe some of his positive contributions to knowledge; the reader will agree that they were important contributions. We have failed to paint a worthy picture of a man who was greater than his work. He cannot live for future generations as John Ray and Gilbert White live; he has left behind no book which will charm by its literary quality after the discoveries it records have become incorporate in the general stock of knowledge. But his personal influence will not die with him. Those who worked beside him and loved him will often be restrained in moments of petulance by a memory of some act of kindness, by a recollection that a great researcher could be as selfless as a child. Any scientific man tempted to measure his own services against their rewards in income or fame, may pause to think how little made this man happy. From Bacot's small circle of intimate friends, the same influence will pass, diluted and weakened, but not destroyed, to others.

...Though much

Will have been lost—the help in strife,
The thousand sweet, still joys of such
As hand in hand face earthly life—
Though these be lost, there will be yet
A sympathy august and pure
Ennobled by a vast regret,
And by contrition sealed thrice sure.

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¹ We are indebted to Professors Martin and Ledingham for permission to use the bibliography compiled for them and printed in the *Brit. Journ. of Exp. Path.* III. 121.



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THE SEGREGATION OF BIOLOGICAL FACTORS IN *B. ENTERITIDIS* (*AERTRYCKE*).

By W. W. C. TOPLEY AND JOYCE AYRTON.

ADDENDUM

To the paper published in *Journal of Hygiene*, XXII, pp. 305—313.

The results of experiments, carried out since this report was submitted for publication and the proof corrected, have led us to alter our views with regard to the nature of those cultures of *B. aertrycke*, which are agglutinated by both type and group antisera.

The conclusions set out in the body of the report cannot, therefore, be accepted in their present form, so far as they deal with the correlation between serological varieties and the degree of faecal excretion.

The questions at issue are being re-investigated in the light of the fresh evidence obtained.

THE SEGREGATION OF BIOLOGICAL FACTORS IN *B. ENTERITIDIS* (AERTRYCKE)

BY W. W. C. TOPLEY AND JOYCE AYRTON.

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University of Manchester.*)

A Report to the Medical Research Council.

(With Plate III.)

IN the preceding report (Topley and Ayrton, 1923 *b*) it has been shown that, if various strains of *B. aertrycke* be fed to mice and the subsequent events observed over a period of 42 days, and if all strains fed to the mice or isolated from their faeces or tissues be examined as regards their type of growth and their agglutination reactions, then the following associations are found to exist between the characters studied. The presence of group antigen is associated with persistent faecal excretion, whether the strain be rough or smooth. Roughness is associated with decreased faecal excretion and a decrease in the percentage mortality, when rough strains are compared with smooth.

In view of these facts it seemed desirable to attempt to define, somewhat more exactly, the relations existing between the various biological factors concerned.

The data already available for the study of this question are not limited to the organisms of the enteric group; and it appears probable that any associations which can be demonstrated within this group will have their counterparts among widely separated groups of bacteria, although there will, of course, be no justification for assuming that associations will always be found between the same biological factors in the different groups.

Arkwright (1921), in his study of smooth and rough strains, was concerned mainly with *B. shiga*, although several other species were studied less completely. All of these belonged to the typhoid-paratyphoid-dysentery group. His studies established the association of a particular kind of colony-formation, "roughness," with flocculation of the bacilli in normal saline; or in salt solutions of lower concentration. This sensitiveness to a relatively low salt-content, when in suspension, was exhibited in another way by the character of the growth in nutrient broth, which was marked by granularity, leading to a heavy deposit with a relatively clear supernatant fluid, often accompanied by the formation of a surface film. In the case of *B. shiga*, Arkwright also found that roughness and smoothness were associated with antigenic differences, as tested by agglutination.

306 *The Segregation of Biological Factors in B. aertrycke*

Schütze (1921), working with organisms of the *Salmonella* group, records observations of a similar kind. He emphasises, however, the lack of correlation between the degree of colonial roughness, the saline instability and the differences in agglutinability when tested by various specific sera. He regards the rough strains as being definitely more cosmopolitan in their serological relationships than the smooth strains.

De Kruif (1921) working with a strain of *pasteurella*, demonstrated the dissociation of this species into a granular and non-granular form, as judged by growth in fluid medium. These differences in the characters of the growth in broth were associated with differences in colony-formation. Far more important, however, was his demonstration that these differences in mode of growth were related to differences in virulence as tested by inoculation into rabbits. The non-granular form was highly virulent, the granular form was almost without effect. De Kruif noted certain small differences between the two types, as regards their agglutination reactions, but these were not very definite and the question was not investigated in detail.

Cowan (1922), working with streptococci, and using rabbits and mice as her test-animals, demonstrated a similar association between smoothness and virulence; and roughness and lack of virulence. Roughness was exhibited by the colonial form and by the character of the growth in broth.

Griffith (1923) demonstrated the existence of the same association between smoothness and virulence, and roughness and non-virulence in the case of the pneumococcus, using mice as his test-animals. Here again the distinction between the character of the growth of the two varieties could be demonstrated either with liquid or solid media. As regards serological relationship, Griffith's findings suggest that the rough form was antigenically a simpler or less complete variant derived from the smooth form. He brought about the change from smoothness to roughness by growing strains of pneumococci in immune serum.

The study by Andrewes (1922) of the antigenic structure of the *Salmonella* group has been referred to in a previous report. It may here be noted that he specifically states that the antigenic differences which he observed were in no way related to roughness or smoothness of the growths.

Both Arkwright and Schütze state that considerable difficulty may be met with in deciding whether a given colony is rough or smooth, and that the association between roughness and a granular growth in broth, and smoothness and a diffuse growth in broth, is not entirely constant. Our own work has dealt only with *B. aertrycke*. With this species we have met with no real difficulty in differentiating between rough and smooth colonies; and the association between roughness on solid media and granularity in nutrient broth has been absolute. This is, however, the case only if we rely entirely on the structure of the surface of the colony, and pay no attention to its border. A smooth colony may vary from a lenticular form, with sharply defined margins and a high degree of translucency, to a much flatter form with deeply

serrated edges, and much more opaque, but in every case the surface is smooth or very slightly granular. The surface of the rough colony is always coarsely granular, giving much the appearance of morocco leather, when viewed with a low-power objective. Plate I shows two smooth and two rough colonies as viewed with a two inch objective and No. 2 ocular.

We have already recorded the fact that rough strains of *B. aertrycke*, when fed to mice, produce a lower percentage mortality than do smooth strains under the same conditions. It was, however, clearly desirable to discover whether the sharp differences between the virulence of rough and smooth strains as tested by direct inoculation into the tissues, which had been demonstrated for pasteurilla by De Kruif, for streptococci by Cowan, and for pneumococci by Griffith, held true in the case of *B. aertrycke*. It was also necessary to determine whether there was any association between the presence or absence of type or group antigen and different degrees of virulence.

We have tested the effect of intraperitoneal inoculation into mice of smooth and rough strains of *B. aertrycke*, using in each case some strains which contained type antigen alone, and others which contained group antigen, alone or combined with type antigen. In the case of the smooth strains, we tested type, group, and mixed varieties. In the case of the rough variants we had not, at the time when these experiments were made, succeeded in isolating pure group strains, so that we compared type with mixed strains.

Table I.

Showing time to death, in days, of mice inoculated intraperitoneally with different strains of B. aertrycke (mutton).

No. of mouse in each series	Dose in c.c. of 18 hours broth culture	Strains							
		Smooth				Rough			
		Type		Group		Mixed	Type		Mixed
		<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	<i>S</i>	<i>K</i>	<i>O</i>	<i>N</i> <i>R</i>
1	0.25	0.75	1	0.75	0.75	0.75	13	1	4 0.75
2	0.25	0.75	0.75	0.75	0.75	0.75	6	3	— 4
3	0.025	1	2.5	0.75	1.5	0.75	12	9	— —
4	0.025	6	0.75	0.75	0.75	0.75	—	6	14 —
5	0.0025	2.5	3.5	3.5	—	4	—	—	— —
6	0.0025	3.5	3.5	0.75	1.5	1	—	—	19 —
7	0.00025	5	6	2.5	12	5	—	—	10 —
8	0.00025	3.5	2	2	7	11	—	—	— —
9	0.000025	3.5	—	11	10	6	—	—	— —
10	0.000025	9	3.5	2	—	7	—	—	— —

— = lived for 21 days.

Table I shows the results obtained in testing nine strains, and they are quite definite. Smoothness is associated with high virulence. Roughness is associated with low virulence. There is no obvious association between the presence or absence of type or group antigen and high or low virulence. As regards strain *N*, the two deaths with small doses are offset by the two survivals with larger doses. They occurred late and cannot be regarded as significant.

308 *The Segregation of Biological Factors in B. aertrycke*

One point may be noted in passing, since we are not aware that similar observations have been reported, and the results in question confirm a previous finding in connection with the persistence of *B. aertrycke* in the tissues after feeding. All inoculated mice were kept under observation for 21 days. At the end of this period all survivors were killed, a post-mortem examination was carried out in each case: and cultures were prepared from the spleen. Of 90 mice inoculated intraperitoneally 30 lived for 21 days. Of these, 27 had been inoculated with rough strains, 3 with smooth. Spleen cultures from these 30 surviving mice yielded pure growths of *B. aertrycke* in 24 cases.

We may also note here that we have never observed a change from smooth to rough, or *vice versa*, in comparing the strains fed or inoculated into mice with the strains isolated from their faeces during life, or from their tissues after death.

Table II.

Showing results of absorbing "type" and "group" sera with "type" and "group" bacteria (B. aertrycke).

Serum	Absorbed with	Titre against			
		Smooth (Type)	Rough (Type)	Smooth (Group)	Rough (Group)
A. Type (Absorbed)	—	3,200	3,200	—	—
"	Smooth (Type)	400	400	—	—
"	Rough (Type)	600	600	—	—
B. Group (Newport)	—	—	—	1,600	6,400
"	Smooth (Group)	—	—	0	0
"	Rough (Group)	—	—	0	0
C. Smooth (Type)	—	25,600	25,600	1,600	6,400
"	Smooth (Type)	400	400	800	1,600
"	Rough (Type)	400	200	200	400
D. Smooth (Group)	—	1,600	1,600	3,200	12,800
"	Smooth (Group)	0	0	0	0
"	Rough (Group)	0	0	0	0
E. Rough (Type)	—	51,200	51,200	100	200
"	Rough (Type)	800	800	0	—
"	Smooth (Type)	800	800	0	100
F. Rough (Mixed)	—	25,600	25,600	1,600	1,600
"	Rough (Group)	6,400	6,400	0	0
"	Smooth (Group)	6,400	6,400	0	0

0 = no agglutination at 1/100.

— = not tested.

Incubation at 55° C. for 2 hours.

It has been noted above that Schütze, in recording his observations on the *Salmonella* group, drew attention to the lack of correlation between the degree of roughness, the saline stability and differences in agglutinability by specific sera. It appeared from our results that, as regards *B. aertrycke*, smoothness and roughness were associated respectively with high and low virulence; while presence or absence of group or type antigen were not associated with different degrees of virulence. It was clearly necessary to determine whether or no variations in antigenic structure were associated with smoothness or roughness. It was already perfectly clear that type and group antigen were present in both smooth and rough varieties. It remained only to determine whether the type and group antigens of the smooth strains were

respectively identical with the type and group antigens of the rough strains. The only difference we had so far noted between the antigenic structure of the smooth and rough varieties was that, while it was quite easy to separate pure type or pure group strains of the smooth variety, we were for many months unsuccessful in isolating a rough strain containing group antigen alone, whereas apparently pure type strains of the rough variant were readily obtained. More recently we have obtained rough strains containing group antigen alone; and we cannot say whether our previous lack of success was due to chance, or to an actual rarity of this combination of biological characters.

In order to determine the identity or otherwise of the antigens of the rough and smooth strains, we carried out the series of absorption tests, the results of which are given in Table II.

It will be noted that, by the time the actual tests were carried out, we had isolated a rough group strain, which was used as one of the test bacterial suspensions. The serum containing group agglutinin, elaborated in response to the inoculation of the group antigen of a rough strain, contained type agglutinin in addition, since at the time the rabbit was inoculated we had only a mixed rough strain at our disposal. The test suspensions used were in all cases formalinised broth cultures, and the same suspension was used throughout for each of the four strains. All essential facts are recorded in the table. Serum A was an absorbed aertrycke serum. Serum B was a newport serum containing a large amount of group agglutinin. Both had been prepared against smooth strains, and were known to differentiate sharply between type and group strains. Sera C, D, E and F were obtained, in each case, by inoculating a rabbit with the antigen specified. As the table indicates, these sera were not absolutely specific, nor were the bacterial suspensions employed for the absorptions strictly specific in their action. The difficulty of obtaining a mass of bacteria sufficient for absorption and containing only one variety of antigen is very great, and there is little doubt that the lack of sharpness in some of the results is due to failure in this respect, and to the inclusion in the suspensions used for immunisation of a proportion of bacteria containing the variety of antigen which we had intended to exclude.

The question at issue is, however, quite clearly answered. The type and the group antigens of the smooth variety are respectively identical with the type and the group antigens of the rough variety.

Taking the results so far obtained, we are faced with the following facts in regard to *B. aertrycke*.

Smoothness is associated with high virulence; as tested by intraperitoneal inoculation into mice, roughness with low virulence.

The presence of group antigen is associated with the phenomenon of persistent excretion in the faeces, its absence with the absence of such excretion.

Smoothness and roughness vary independently of the presence or absence of type or group antigen.

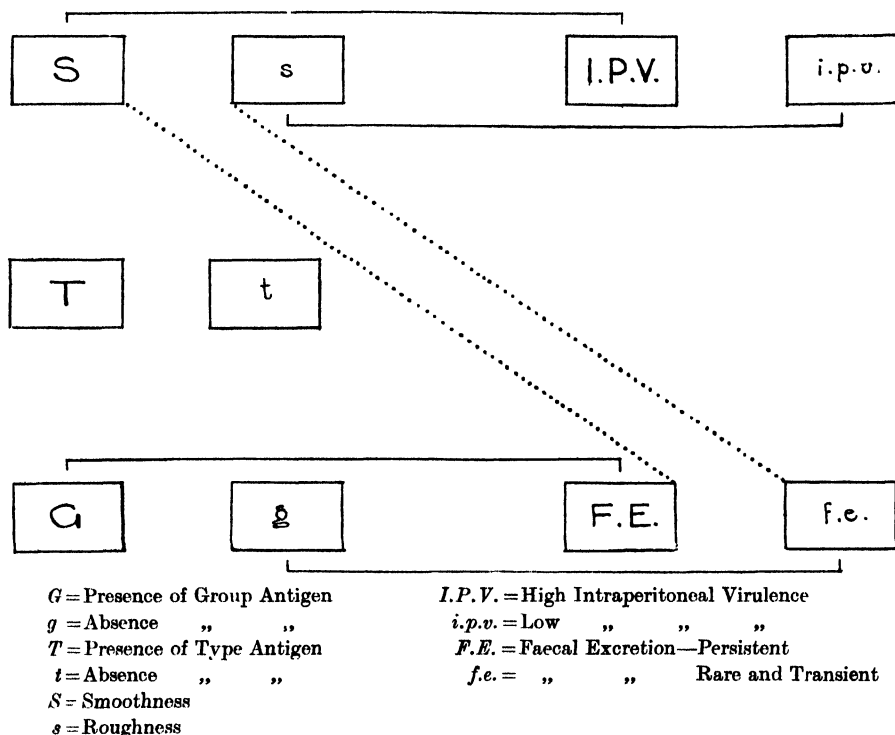
310 *The Segregation of Biological Factors in B. aertrycke*

Although the association of group antigen with persistent faecal excretion is as close among rough strains as among smooth, yet there appears to be some other factor which determines a diminished faecal excretion, when rough strains as a whole are compared with smooth strains as a whole.

It seems difficult to avoid the conclusion that a segregation of biological factors may occur, when *B. aertrycke* undergoes division; that, when such segregation takes place, there is a definite association or linkage between certain of these factors; that the factor which determines smoothness is either identical with, or is inseparably linked to, the factor which determines high intraperitoneal virulence; and that the factor for group agglutinin is similarly associated with the factor which determines persistent excretion in the faeces.

Diagram I indicates the linkages which we may suppose to exist. There appears to be some reason for believing that roughness is due to the loss of

Diagram I.



some character which is present in the smooth strains. We have indicated this probability in the diagram by using a symbol for absence or decrease, as in the case of the other factors considered. We have indicated the linkage between smoothness and faecal excretion by a dotted line, in place of the continuous line used in the other cases, in order to emphasise that the association is independent of that existing between the other factors, and appears

to be of a different order. It will be noted that we have so far obtained no evidence of any linkage affecting the type antigen.

It remains to express some opinion as to the number of different varieties of *B. aertrycke*, which may result from such segregation of factors. We have mentioned, in the preceding report, that we are disposed to recognise a variety of *B. aertrycke* in which both the type and group antigen are fully developed in each bacillus. If we are correct in this view, such a strain would clearly represent the complete organism from which the other variants were derived by loss of certain factors.

The evidence which leads us to support this view is largely based on the frequency with which we have encountered subcultures from single colonies, which agglutinate with both type and group sera. It is frequently the case that 20 or more colonies from one plate will show this result, while, when pure type and pure group strains have been isolated from the same plate, the proportion of mixed colonies has often been very small.

There are, however, two observations which appeal to us with particular force. One concerns the agglutination results obtained with strains isolated from the tissues of mice which have been inoculated with group or type strains, or fed on such strains.

Of 382 strains isolated from mice inoculated with type strains, 330 reacted as type, 44 as mixed, and 8 as group. Of 313 strains isolated from mice inoculated with group strains, 231 reacted as group, 81 as mixed, and 1 as type. Of 159 strains isolated from mice fed on type strains, 107 reacted as type, 50 as mixed and 2 as group. Of 143 strains isolated from mice fed on group strains, 83 reacted as group, 60 as mixed and none as type. This is not the distribution which would be expected on the assumption that the strains which reacted as mixed were derived from colonies which owed their mixed character to the chance association, in the formation of the colony, of a group and a type bacillus.

The other piece of evidence is gathered from a consideration of the results obtained by applying agglutination tests to considerable numbers of strains isolated from the faeces of individual mice on repeated examination.

In one experiment (*H*), in which a series of mice were fed on a smooth group strain of *B. aertrycke*, 20 strains isolated from six specimens of faeces, collected from one mouse, over a period of 16 days, were tested by agglutination. All reacted as pure group strains. In the case of another mouse of the same batch, 18 of 23 strains isolated from the faeces reacted to both group and type sera. Specimens from this mouse were examined over a period of 27 days, and while the first two specimens gave pure group strains, these were later replaced by mixed strains, and mixed strains alone were isolated from the last three specimens examined. In the whole experiment no type strain was ever isolated.

In another experiment (*S*) in which a batch of mice were fed on a strain which agglutinated with both type and group sera, 23 positive specimens of

312 *The Segregation of Biological Factors in B. aertrycke*

faeces were obtained from 5 mice. From these 23 specimens 88 strains were isolated and tested by agglutination: 86 of these were agglutinated by both type and group sera: 2 reacted as pure group strains, none as pure type strains. Many other observations of a similar kind could be cited.

It seems impossible to explain such figures as the result of chance admixture of type and group bacilli in the same colony¹. To do so in the case of Exp. *S* we must accept the view that chance selection has resulted, on 86 occasions, in our picking colonies which have been formed from the development of a type and a group bacillus lying in accidental juxtaposition, while we have only twice picked a pure group colony, and have not once picked a pure type colony. We cannot invoke any theory of symbiosis, for

Diagram II.

	Type +	Group +	Behaves as Type - Group +
Smooth	Type +	Group -	{ Intraperitoneal virulence high. Excretion in faeces after feeding rare and transient
	Type -	Group +	{ Intraperitoneal virulence high. Excretion in faeces after feeding frequent and persistent
	[Type -	Group -]?	
Rough	Type +	Group +	Behaves as Type - Group +
	Type +	Group -	{ Intraperitoneal virulence low. Excretion in faeces after feeding rare and transient
	Type +	Group +	{ Intraperitoneal virulence low. Excretion in faeces after feeding frequent and persistent but less so than with the corresponding smooth variety
	[Type -	Group -]?	

we know (a) that pure group strains are readily excreted as such in the faeces, (b) that, although pure type strains are seldom excreted, yet, when such excretion occurs, they are not accompanied by group or mixed forms, and (c) that when we purposely feed to mice a mixed culture, known to contain both type and group bacilli, strains of both of these types are isolated from the faeces, though the group strains greatly predominate.

Finally, we have, in the preceding paper (Topley and Ayrton, 1923 *b*) brought forward evidence which shows that pure type strains are seldom excreted in the faeces. The results of Exp. *S*₆ could only be explained as due to the chance admixture of type and group bacilli, if the two varieties were excreted in the faeces with equal readiness.

Consideration of these results would lead on naturally to a discussion of the extent to which variations in antigenic structure occur in the intestine or in the tissues. This question has been briefly referred to in the preceding report and we hope to consider it in more detail in future communications.

¹ "It will clearly be impossible to distinguish, with the technique employed, between a colony, in which the individual bacilli contain both type and group antigens, and another colony, in which the bacilli contain only one kind of antigen, but are particularly liable to give rise to bacilli containing the other kind of antigen, in both subcultures. In both cases we should obtain agglutination with both group and type antisera."

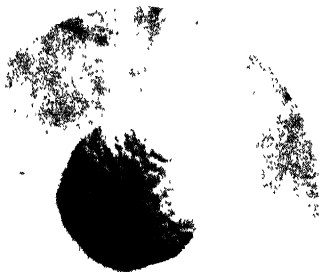


Fig. 1



Fig. 2



Fig. 3



Fig. 4

Figs. 1 and 2. Rough colonies (2 inch obj. and No. 2 ocular).
Figs. 3 and 4. Smooth „ „ „ „

At the moment we are concerned only with the question of the existence of a complete or mixed antigenic variety of *B. aertrycke*.

We should, for the reasons given, be disposed to recognise at least six varieties of this organism, and probably eight. We have indicated the nature of these varieties in Diagram II, and have noted in each case the characteristics which distinguish the behaviour of the six varieties we have studied, when functioning as parasites with the mouse as host. The two varieties of *B. aertrycke* which contain neither type nor group antigen, if such varieties exist, have not been studied as regards their behaviour as parasites. As noted elsewhere, we have, on several occasions, met with strains of *B. aertrycke* which react neither to type nor to group sera; but whether their inagglutinability is due to absence or marked deficiency of the corresponding antigens, or to some quite different cause, it is impossible to say until we have more facts at our disposal.

DISCUSSION.

There is little to add to what has been said above, but we should wish to emphasise one point. We have purposely employed terms drawn from Mendelian sources. The biological factors discussed must have a material basis, and the suggestion that the variations observed are due to an unequal distribution of the substances concerned, at the moment when division of the bacillus occurs, is so obvious, that it may well be provisionally accepted as a working hypothesis. Such acceptance does not imply any specific view as regards the structure or mode of reproduction of the bacilli. We know that multiplication by binary fission occurs, and this form of growth gives ample opportunity for such a segregation as we have suggested.

The conclusions arrived at have been set out in the body of the report and need not be repeated.

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NOTES ON THE SPORULATION OF *B. SPOROGENES* AND OTHER ANAEROBES

(A REPORT TO THE FOOD INVESTIGATION BOARD)

BY F. P. G. DE SMIDT, M.R.C.S., L.R.C.P., D.P.H.

(*From the Pathological Department, Manchester University.*)

(With 1 Chart)

IN the course of experiments on the cultivation of anaerobic bacilli, it was found advisable to be able to calculate with some certainty on the production of spores by the strains of anaerobes worked with. Observations were accordingly made of the conditions of cultivation by which sporulation appeared to be influenced. It was evident that in the case of *B. sporogenes* and other well-known types observed, the proportion of spores produced within a given period varied markedly in response to variations in the same factors that mainly influenced the rate of growth in cultures, viz. concentration of food material, incubation temperature, and reaction of medium. With the exception of cultures in media containing added carbohydrate, it was evidently also the rule that sporulation was most active in cultures commencing under conditions most favourable to rapidity of vegetation.

These points are perhaps too well known to need further emphasis, but a note on one aspect of their biological significance may not be out of place. If we consider two communities of a type of anaerobe—(*A*) multiplying slowly in poor environment, and (*B*) multiplying rapidly in a rich environment, it is evidently of advantage to the type that sporulation should be the more profuse in community (*B*); for then on the death of both communities the greatest number of spores are ensured to propagate the race.

It is easily conceivable that thorough study of sporulation in anaerobes and the conditions governing it should furnish valuable knowledge especially perhaps in connection with the preservation of foods. Many notes on sporulation may be found in the literature dealing with type characters of anaerobes, and at least one series of experiments on the sporulation of a particular type has been published (Fitzgerald, 1911). But no work devoted to a general treatment of the subject has been met with; nor does any method appear to have been described for observation on an arithmetical basis.

Experiments were therefore made with a view to testing a simple method permitting of numerical estimation of the proportion of spores present in cultures. The method was considered to give sufficient accuracy to enable practical conclusions to be drawn; and it was accordingly applied to a preliminary investigation of the more important factors in cultivation that

influence sporulation. Although very little, if anything, of value may have been added to our knowledge in the following notes, it is hoped that these methods of observation may be useful as a basis to others wishing to carry out more important work on the subject.

METHOD OF ENUMERATING SPORES.

The principle is simply that of "projecting" microscopic fields on to squared paper, and marking off spores, spore-bearing bacilli, and vegetative bacilli on small areas to facilitate counting. This was done by means of an ordinary camera lucida attached to the eye-piece of the microscope in the

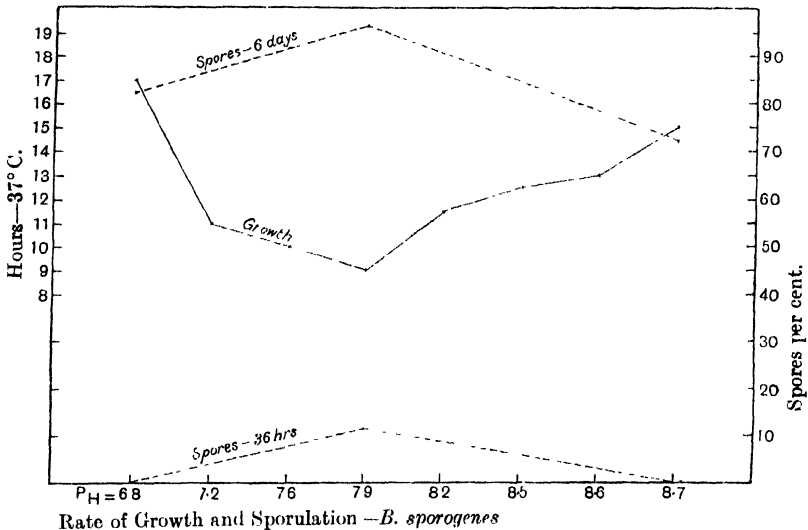


Chart 1

horizontal position. Graph paper was used printed in 1/10 in. squares, and further divided with black ink into 1/2 in. squares. It was arranged that a microscopic field viewed in projection on the graph paper embraced 36 of the 1/2 in. squares. Film preparations for counting were made on cover glass slips, which were mounted in a drop of 1-5 dilution in water of the ordinary 1 per cent. methylene blue stain; to the diluted methylene blue sufficient caustic soda had been added to procure immediate intense staining of healthy bacilli. After mounting, the edges of the cover slip were sealed with vaseline. It is noteworthy that if film preparations are over-fixed by slightly prolonged heating, mature spores are stained with considerable intensity by the above or other ordinary methods: as I believe, first pointed out by P. N. Panton (1908). It is unnecessary, however, for the present purpose that the spores should be stained at all.

In using the camera lucida, the lighting must be controlled so that the reflection of the microscopic field seen in projection on the squared paper is not too bright for the process of marking to be clearly visible.

316 *Sporulation of B. sporogenes and other Anaerobes*

Such microscopic fields were chosen that at least 300 organisms were available for counting. Spores and spore-bearing bacilli were dotted off with red ink, vegetative bacilli with black ink. A bacillus was not marked as a spore-bearer unless that spore was mature enough to be at once recognisable either by the presence of a swelling, or by its "refractility," in types where the spore produces no swelling.

In the complete chart, the red and black dots, representing spores and bacilli, were counted, and the proportion of spores recorded as a percentage of the total organisms enumerated. At first, results were expressed as a "sporulation index" represented by the quotient obtained by dividing the number of spores by the number of bacilli. Thus with 92 spores and 318 bacilli, the "sporulation index" is 0.3 approximately. This device was not found to be generally convenient. The error involved in the method was gauged by estimating the proportion of spores counted in homologous films and cultures.

METHOD OF CULTIVATING ANAEROBES FOR SPORE-ESTIMATION.

Cultures for sporulation were inoculated in the broth known as P 3, which is a filtered extract of pancreas, first prepared by G. S. Graham-Smith, by a method described in another communication (de Smidt, 1923). P 3 affords an exceptionally rich nutrient medium for the types of anaerobes experimented with, so that if needed a poor nutrient environment can be readily produced by dilution.

The methods of obtaining anaerobic cultures were based on McIntosh and Fildes' invaluable method for surface cultivation in single tubes, depending for the removal of oxygen, on the use of hydrogen and "palladium-asbestos-wool" (McIntosh and Fildes, 1917).

In earlier experiments, broth containing 0.5 to 1.0 per cent. of glucose was used, and was strained and cooled immediately before inoculation. Later, plain broth, containing no added carbohydrate or solids, was rendered sufficiently oxygen-free for anaerobic growth by means of a simple procedure of re-heating in an oxygen-free atmosphere -- more convenient than, and probably as reliable as the more cumbersome methods depending upon exhaustion apparatus.

Details of the method are given below.

Cultures were made either in duplicate or in separate anaerobic tubes, the contents of which were thoroughly mixed together before spore-enumeration; or they were prepared in small tubes each containing 2 c.c. of broth, which were incubated within large anaerobic containing tubes each holding four or five cultures.

EXPERIMENTAL ERROR IN ENUMERATING SPORES.

In these and all subsequent experiments, the broth cultures were subjected to prolonged shaking before preparing films for spore counting.

A. *Estimation of spores in films from same culture*—*B. sporogenes*.P 3 broth, reaction $pH = 7.5$. Incubation 5 days at $37^{\circ}C$.

Total organisms counted	Spores	Spores %
334	274	82
370	314	85
417	345	83
380	316	83
385	301	78

Error = 7

B. *Estimation of spores in films from uniform cultures*—*B. sporogenes*.

One film from each of five cultures, uniformly inoculated with a loopful of spore suspension transferred to 2 c.c. of P 3 broth, reaction $pH = 7.5$. Cultures in same anaerobic container, 96 hours at $37^{\circ}C$.

Total organisms	Spores	Spores %
453	76	17
338	70	21
410	72	22
449	78	17
586	63	11

Error = 11

The importance of the error was reduced in the following experiments by repeating them as many times as was considered necessary for reliable conclusions to be drawn from the results.

RELATION OF SPORULATION TO RATE OF GROWTH.

Experiments were made to gauge the influence on sporulation of variations in certain primary factors that govern the rate of growth of organisms in culture. Throughout, except when otherwise mentioned, tubes containing 2 c.c. of broth were inoculated with one standard loopful of a shaken suspension of spores in pure culture.

I. *Influence of reaction of medium*—*B. sporogenes*.

(a) P 3 broth, containing 1 per cent. glucose, in duplicate cultures, incubated 96 hours at $37^{\circ}C$.

Reaction	Spores
$pH = 6.4$	1.8 %
$pH = 7.7$	77.7
$pH = 8.7$	58.0

(b) P 3 broth, plain, cultures in same anaerobic container, incubation (1) 6 days at $37^{\circ}C$, (2) 36 hours at $37^{\circ}C$.

Reaction	(1) Spores	(2) Spores
$pH = 6.8$	82.8 %	Nil
$pH = 7.9$	96.8	11.5 %
$pH = 8.7$	73.6	Nil

The accompanying chart shows the rate of growth of a strain of *B. sporogenes* in P 3 agar at various hydrogen ion concentrations. The curve is based on the

318 *Sporulation of B. sporogenes and other Anaerobes*

times of appearance of growth in a duplicate series of stab cultures. Disregarding any discrepancies arising from slight variations in quality of the samples of P 3 used, two of the above results are superimposed for comparison on the rate of growth curve.

II. *Influence of temperature*—*B. sporogenes*.

P 3 broth, containing 1 per cent. glucose, reaction $pH = 7.8$, in duplicate cultures; (1) incubated 96 hours at $37^{\circ}C.$, (2) incubated 18 hours at $37^{\circ}C.$ then transferred to electric incubator at $25^{\circ}C.$ for 88 hours.

	Temperature	Spores
(1)	$37^{\circ}C.$	69.3 %
(2)	$25^{\circ}C.$	2.9

Rate of growth was judged by observing the appearance of similar cultures after 24 hours' incubation at $37^{\circ}C.$ and $25^{\circ}C.$

	Temperature	Growth	Spores, 96 hours
(1)	$37^{\circ}C.$	Turbid, frothing	Very abundant
(2)	$25^{\circ}C.$	Opalescence	Scanty

III. *Influence of concentration of food material*—*B. sporogenes*.

Comparison was made of sporulation in P 3 broth diluted with water, and full strength P 3 broth. Cultures were inoculated both with small and very heavy sowings.

(a) P 3 broth, reaction $pH = 7.7$, (1) 20 per cent. P 3 and 1 per cent. glucose, (2) full strength P 3 and 1 per cent. glucose. Duplicate cultures inoculated with one loopful of spore suspension, incubated 72 hours at $37^{\circ}C.$

	Medium	Spores, 72 hours	Growth, 20 hours
(1)	P 3 20 %	0.9 %	Opalescence
(2)	P 3 full strength	12.5 %	Turbidity, gas

(b) P 3 broth, reaction $pH = 7.7$ in strengths (1) 15 per cent. P 3 and 0.5 per cent. glucose, (2) full strength P 3 and 0.5 per cent. glucose. Duplicate cultures inoculated with one loopful of spore suspension, incubated 96 hours at $37^{\circ}C.$

	Medium	Spores, 96 hours	Growth, 20 hours
(1)	P 3 15 %	Nil	Opalescence
(2)	P 3 full strength	79.2 %	Turbidity, gas

(c) P 3 broth, reaction $pH = 7.7$ in strengths (1) 20 per cent. P 3 and 1 per cent. glucose, (2) full strength P 3 and 1 per cent. glucose. Duplicate cultures in 2 c.c. broth inoculated with 0.5 c.c. of a vigorously growing 20 hours' broth culture. Incubated 72 hours at $37^{\circ}C.$

	Medium	Spores, 72 hours	Growth, 20 hours
(1)	P 3 20 %	0.4 %	Cloudiness
(2)	P 3 full strength	16.6 %	Turbidity, gas

P 3 broth, reaction $pH = 7.7$ in strengths (1) 15 per cent. P 3 and 0.5 per cent. glucose, (2) full strength P 3 and 0.5 per cent. glucose. Duplicate cultures in 2 c.c. broth, inoculated as above; incubated 96 hours at $37^{\circ}C.$

Medium	Spores, 96 hours	Growth, 20 hours
(1) P 3 15 %	Nil	Cloudiness
(2) P 3 full strength	87.7 %	Turbidity, gas

On the assumption that sporulation proceeds in relation to the exhaustion of food material, it might be supposed that dilute broth should become exhausted earlier than full strength broth, and the above results should be reversed. It is probable, however, that the rich medium is more rapidly used up than the poor, by reason of the rate of growth in the former being enormously greater in proportion.

IV. Influence of carbohydrates.

It is well known that with certain types of anaerobes sporulation is less profuse in media to which glucose has been added. The following experiment with *B. sporogenes* shows that sporulation is inhibited by increasing the quantity of glucose added to broth. P 3 broth, 90 per cent., reaction $pH = 7.8$, in duplicate cultures containing (1) 0.5 per cent. of glucose, (2) 2 per cent. of glucose. Inoculated with one loopful of spore suspension, incubated 96 hours at 37° C.

	Glucose	Spores
(1)	0.5 %	60 %
(2)	2.0 %	9 %

Inhibition of sporulation by carbohydrate may be due to production of acid by fermentation, with the result that the reaction of the medium is rapidly altered to an extent that greatly lessens the rate of growth.

Fermentation of carbohydrates in its effect on sporulation. The following experiments indicate that sporulation is inhibited only when the carbohydrate present is fermented by the type of organism concerned.

Medium. P 3 broth, 50 per cent. strength, coloured with litmus, containing (1) 1 per cent. of glucose, (2) 1 per cent. of lactose, (3) no added carbohydrate. The plain dilute broth did not contain sufficient sugar to be demonstrable by Fehling's reagent.

Types of anaerobe. *B. sporogenes*, which ferments *glucose*, but not *lactose*; McIntosh's *Type 3 c* ("round spored V. Hibler") which ferments neither of these sugars; and *B. butyricus* (Adamson) which ferments both *glucose* and *lactose*.

Cultures in 2 c.c. of above media, uniformly inoculated with spore suspension, and incubated in same anaerobic container for 6 days at 37° C. Acid reactions when present were strongly marked in 24 hours.

(a) *B. sporogenes*.

Reaction	Glucose	Lactose	Plain broth
Spores	Acid	Unchanged	Unchanged
	Nil	46 %	42 %

(b) *Type 3 c*.

Reaction	Unchanged	Unchanged	Unchanged
Spores	63.2 %	61 %	60.9 %

(c) *B. butyricus*.

Reaction	Acid	Acid	Unchanged
Spores	Nil	Nil	70.3 %

320 *Sporulation of B. sporogenes and other Anaerobes*

Error in sugar experiments. Using Type 3 c, five cultures were prepared in 2 c.c. of 50 per cent. P 3 broth containing 1 per cent. of glucose: these were incubated in the same anaerobic container, and the spores estimated after 6 days at 37° C. Results: 63, 65, 66, 72 per cent. respectively.

The greatest difference between two counts = 9 per cent.

Constancy of reaction in sugar-free broth cultures. As shown above, no alteration of reaction was indicated by litmus in 50 per cent. P 3 broth containing no sugar.

To decide whether any slight or gradual change in hydrogen ion concentration occurred in cultures in plain broth, to be considered as a possible factor influencing sporulation, the hydrogen ion concentration of a sample of P 3 broth was estimated before and after prolonged cultivation of *B. sporogenes*.

A sample of P 3 broth containing no added carbohydrate was adjusted to $pH = 7.4$ by the colorimetric method. Of this two tubes containing 10 c.c. were heavily inoculated with *B. sporogenes* and became turbid with growth within 20 hours at 37° C. Incubation at this temperature was continued for 9 days, when examination of films showed a large majority of free spores, the remainder consisting of bacilli bearing mature spores, with occasional sporeless bacilli of degenerate appearance. The cultures were centrifugalised, the clear fluids pipetted off and mixed, and the hydrogen ion concentration again taken. The result was $pH = 7.4$. No difference was appreciable by the colorimetric method between the cultivated broth and a sterile sample of the original broth which had been steamed, for the same length of time (10 mins.) as the former before inoculation, and incubated in sealed tubes for the same length of time along with the cultures.

V. *Problem of the determining factor in sporulation.*

The foregoing experiments indicate that sporulation is dependent upon rate of growth, and support the view that, provided no disturbing factor arises, the more vigorously a number of bacilli inoculated in a culture are enabled to multiply, the larger will be the proportion of spore-bearing individuals present within a given period. The growth of an organism, for example *B. sporogenes*, in a nutrient medium causes alteration of that medium. The alteration consists broadly in (1) exhaustion of the food material required by the organism, (2) chemical change due to products of growth of the organism. The external factor that determines the production of a spore by a bacillus must be, normally, the stimulus of one or the other of these conditions of its environment. Abnormal influences such as the introduction of free oxygen into an actively growing anaerobic culture, may and probably do stimulate sporulation. Alteration of hydrogen ion concentration, such as is caused by acid fermentation of carbohydrate, has been shown to be an inimical factor. No appreciable alteration in the hydrogen ion concentration of a sugar free medium is brought about by the vigorous growth in it of *B. sporogenes* up to the point at which vegetation has ceased and only free spores remain. Alteration in hydrogen

ion concentration can therefore be eliminated as a possible stimulus. The determining cause must therefore be sought for either in the effect upon the bacilli of a diminution of their nutriment; or in the presence in sufficient amount of some chemical body introduced into the environment by means of their own growth therein.

PRODUCTS OF GROWTH IN CONNECTION WITH SPORULATION.

Attempts were made to determine whether spent broth in which *B. sporogenes* had grown vigorously, contained any element capable of stimulating sporulation in the same organism. Broth cultures 7 days old, in which sporulation had reached a very advanced stage were centrifugalised without interrupting anaerobiosis. From the clear top fluid both aerobic and anaerobic tubes of medium were inoculated to control its sterility. These cultures were negative. Of the spent broth, volumes of 0.25 c.c. were added to two of four 24 hours' broth cultures of *B. sporogenes* in 2 c.c. of medium. To the remaining two cultures, the same volumes of sterile boiled water were added. The transfers of spent broth and water were performed rapidly, and the cultures treated were at once rendered anaerobic again, so that no oxygen sufficient to interrupt growth was introduced. A further set of cultures was treated with 0.5 c.c. volumes of spent broth and water. Both sets of cultures were incubated at 25° C. for 6 days, after which film preparations were examined for spores. Unfortunately, the enumeration method could not be used here, and ordinary judgment was relied upon for comparing results.

In both experiments it was found that a moderate proportion of spores had appeared in all four cultures; but no difference could be discovered between the films from the cultures treated with spent broth and those to which water had been added. It was concluded therefore that the spent broth had exercised no influence on the ordinary progress of sporulation in the cultures treated with it.

REINOCULATION OF CULTURES WITH THE SAME STRAIN OF ORGANISM.

Experiments were made to obtain evidence of the relation of sporulation to exhaustion of food material, as judged by the capacity of the medium to support growth at various stages of a culture therein. A number of stab cultures were inoculated with *B. sporogenes* in large deep tubes of nutrient agar, and incubated at 37° C. At intervals of 48 hours, secondary inoculations of the same organism were made in stabs parallel to the original in each culture in succession. At the same time, film preparations from the original inoculation were examined for spores.

After 48 hours, spores amounted to about 30 per cent.; secondary inoculation yielded a visible growth. At 96 hours, about 70 per cent. of spores were present; secondary inoculation showed no visible growth.

It is conceivable that when bacilli of spore-bearing type are fully supplied with nutrient material, and with the conditions that enable them to make

322 *Sporulation of B. sporogenes and other Anaerobes*

use of it, all energy derived from the food is directed towards vegetation. When the food supply fails by exhaustion, insufficient energy is obtainable for multiplication, and what remains is directed towards spore-formation.

It has been shown that of two communities in the same environment, one at the favourable temperature of 37° C. grows and spores vigorously, while the other at the unfavourable temperature of 25° C. grows and spores feebly. On the above tenets, it might be supposed that at the low temperature, the bacilli, being unable to make full use of their food, should direct what energy is available to producing spores. As shown, however, sporulation in these conditions is greatly delayed, probably because multiplication proceeds, however slowly, as long as sufficient energy can be got to maintain it. At length the stage is reached for each individual bacillus when food energy is so far deficient that division is impossible; sporulation then commences. The cycle is thus precisely the same as that proceeding at the favourable high temperature, but covers a longer period.

Similarly, where equal numbers of bacilli are introduced into a poor and a rich environment respectively, other conditions being equal, multiplication is proportionately vastly more rapid in the rich medium; with the result that the stage is reached earlier at which food energy is insufficient for multiplication; but is still sufficient for spore production.

ABNORMAL INFLUENCES IN SPORULATION.

By abnormal influences is implied those of conditions which do not as a rule arise in the environment of a community of anaerobic bacilli. One of these perhaps is the intermittent exposure of the organisms to free oxygen.

Experiments in this direction were made by aerating broth cultures of *B. sporogenes* at intervals of 24 hours by means of a two-way syringe and a sterile pipette passing through the plugs of the culture tubes. The cultures so treated were incubated at 25° C.: it was found that up to a period of 5 or 6 days sporulation appeared to be markedly increased as compared with similar cultures not aerated. No enumerations of spores were made in these experiments, and their results are therefore not considered conclusive. The probability that spores may be produced in response to exceptional conditions does not interfere with the conclusion that sporulation normally proceeds in response to failure of the food supply; among higher organisms, certain processes such as germination of the ovum may work independently of normal stimuli, or in response to artificial ones.

It is not presumed that the experiments here described are sufficient for their purpose. Possibly the determining factor in sporulation may be a combination of exhaustion of nutriment with the arrival at a certain concentration of growth products. Nor is any account taken of physical and physiological factors that may be at work. The bearing of specific differences in the behaviour of types must be considered. For example, a strain of *B. tertius* produced spores so rapidly in P 3 broth that within 24 hours

scarcely a sporeless bacillus remained; while *B. sporogenes* under the same conditions showed few signs of spore-swellings in this time.

There is no doubt that the problems presented by the sporulation of anaerobes are thoroughly worthy of investigation: and are such as to tax to the utmost the resources of the biochemist and biologist. Not the least of these is the problem offered by the spore itself, which enclosed within a wall of extraordinary powers of resistance to physical and chemical agents, is yet so delicately sensitive to external conditions that it germinates at once if placed in contact with a suitable nutrient surface.

METHODS OF ANAEROBIC CULTIVATION IN BROTH.

(1) *Single tubes* were rendered anaerobic by McIntosh and Fildes' method (1917), depending upon hydrogen delivered from a Kipps apparatus or modification, and palladium-asbestos-wool for removal of residual oxygen.

With glucose broth, the tubes of medium were inoculated after steaming and cooling in the usual way.

With plain broth, the tube was first steamed for 10 minutes, then while hot, rendered anaerobic and sealed, and the junction of stopper and tube painted with collodion. With the palladium-wool capsule still hot, the tube was replaced in boiling water for 5 minutes. It was then transferred to cold water without unsealing. When cold, the tube was unsealed, the broth rapidly inoculated without shaking and the tube at once rendered anaerobic and sealed again, painting with collodion as before.

When inoculating cultures, the corks as well as the mouth of the tubes were flamed for a second in the bunsen burner.

The capsule referred to consists of a small piece of "palladium-wool" folded in a single layer of copper gauze for safety, and to prevent pieces dropping into the culture.

Further details of McIntosh and Fildes' well-known method are given in their report as above.

(2) *Small tubes under the same anaerobic conditions.* Large, stout glass tubes 8 inches in length by about 3 centimetres of *internal* diameter, were used as containers. These, as described by McIntosh and Fildes for small single tubes, are fitted with rubber bungs bored with two holes to fit quill tubing. Two short pieces of quill tubing, drawn out pipette-wise at one end, are inserted into the holes, after lubrication with a little vaseline, by passing them point first through the bottom of the bung, and forcing them through by pressure on a table.

After insertion, one of the pipettes is bent to a right angle for the hydrogen outlet. The pipettes can be easily removed when necessary by pushing them out from below with a metal rod. A small capsule about 1 in. square of palladium-asbestos-wool folded in a single layer of gauze is fastened to the centre of the bottom of the bung with a pin pushed in while red hot. The culture tubes were 11 centimetres long by about 12 millimetres in *external*

324 *Sporulation of B. sporogenes and other Anaerobes*

diameter. Containing 2 or 3 c.c. of plain broth, and tightly plugged with wool, these are steamed for 10 minutes to expel air. Four or five at once are then transferred while hot into the container, which should contain a little hot water sufficient to immerse the ends of the culture tubes, and act as a conductor of heat. The container is at once rendered anaerobic, sealed, painting the bung at its insertion with collodion, and then the whole placed in boiling water while the capsule is still hot; there is no danger of the bung being forced out. After some minutes' steaming, the container is transferred to cold water without unsealing. When cold, the sealed tips of the pipettes are cut off, and the bung removed; the culture tubes are extracted by their plugs with forceps, quickly inoculated, and replaced, and anaerobiosis again set up in the container. The whole procedure, after unsealing the container, must be as rapid as possible without shaking the culture tubes. Although this form of anaerobic container is best suited for surface cultivation in small tubes of agar, the method described here is convenient and fairly rapid, besides being inexpensive; and it was found to be sufficiently dependable for the experiments detailed above.

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AN APPARATUS FOR ANAEROBIC PLATE CULTIVATION IN HYDROGEN FOR SEPARATE PETRI CAPSULES

(A REPORT TO THE FOOD INVESTIGATION BOARD)

By F. P. G. DE SMIDT, M.R.C.S., L.R.C.P., D.P.H.

(With Plate IV.)

A NUMBER of methods have been described for anaerobic cultivation in separate plates. The principle offers certain advantages over the cultivation of a number of plates in the same anaerobic container, which has to be opened whenever it is desired to inspect any particular culture; and it is useful where anaerobic plate cultures are needed only occasionally or in small numbers.

A few methods are well known and have been widely used. These, however, depend upon alkaline pyrogallate for absorption of oxygen; and this principle is not always dependable, being especially liable to fail with light inoculations of pure culture of anaerobes.

A method was accordingly devised for single anaerobic plate cultivation in an atmosphere of hydrogen; in this the residual oxygen is removed by means of a heated capsule of asbestos-wool impregnated with carbonised palladium, after the method of McIntosh and Fildes.

A very perfect degree of anaerobiosis is thus quickly and conveniently obtained.

CONSTRUCTION OF APPARATUS.

The Petri capsule-holders used are made from the flanged metal tops of canisters in which jam and other commodities are commonly sold; following Henry's well-known device for single alkaline pyro-gallol plates. These canister tops consist of a flanged metal rim, with a central plate which must be removed to get at the contents of the canister. For the present purpose the flanged rim is cut off, and the central plate is soldered in position so as to be air-tight. The result is a shallow circular metal dish bordered with a wide groove which forms the Petri capsule plate-holder. To the back of this are soldered two short lengths of "white metal" piping of about 12 mm. external diameter. These are blocked at their inner ends, and their open ends project about half an inch beyond the rim of the plate-holder. Two small holes bored in the central portion of the plate-holder correspond to holes bored in the metal pipes close to their blind ends. The soldering of the pipes must be thoroughly done, so that the communications between the pipes and the interior of the plate-holder are perfectly air-tight.

Figs. 1 and 2 are photographs of the back and front, or external and internal aspects, of a plate-holder, and show its construction clearly. The

inner surface of the holder is japanned black to afford a dark background for colonies. Two sizes of plate-holder were used; one to fit the smaller half of a Petri capsule, and one to fit the larger half or cover. One Petri capsule was thus made to serve for two cultures. It was found easier to choose Petri capsules to fit plate-holders, than to choose tins whose flanged tops would fit Petri capsules. This is because Petri capsules of the same catalogued size are, nevertheless, variable in diameter; whereas tin canisters are made very accurately to certain measurements. Petri capsules were used in which the external diameter of the smaller halves was about 8 centimetres; these were catalogued as 3½-inch size, and both halves could usually be fitted to plate-holders made from easily obtained sizes of canister. To the open ends of the metal pipes pieces of glass tubing drawn out pipette-wise are attached by means of thick-walled (semi-pressure) rubber tubing. One of these pipettes—that for the hydrogen outlet—contains a cartridge about 1½ inches long of “palladium-asbestos-wool” rolled in a single layer of copper gauze. The palladium-asbestos-wool (McIntosh and Fildes, 1917) can be obtained from Messrs Baird and Tatlock. It is best to lubricate the rubber tubing connections with a little vaseline.

Figs. 3 and 4 show the apparatus complete with colonies of *B. tetani* and *sporogenes*.

METHOD OF USE.

The separate halves of the Petri capsule are covered with the plain lids of cylindrical tin canisters which can be easily obtained of suitable sizes. They are sterilised in these covers and charged with nutrient agar in the usual way. Before inoculation, to secure discrete colonies, the agar plate should be dried for quite 2 hours in the incubator in the usual way by tilting it bottom upwards against the rim of its cover.

Two pieces of fresh plasticine are rolled into long “worms”; one of these is pressed well into the groove of the plate-holder so as to fill it completely. After inoculation and removing its tin cover, the plate is pressed firmly into its bed of plasticine, care being taken to apply pressure at the sides, and not near the centre of the plate, to avoid risk of cracking the glass. The second “worm” of plasticine is then applied to the junction of plate and holder, and thoroughly welted in and smoothed round to effect a perfectly air-tight connection. Dryness of the glass is essential for this, and may be secured if necessary by wiping with a cloth moistened with spirit.

The apparatus is rendered anaerobic by means of hydrogen delivered from a Kipps' apparatus. The inlet pipette—that which does not contain the cartridge—is inserted until it engages into a length of pressure tubing attached to the Kipps. The gas is turned on full, ignited at the outlet pipette, and allowed to blow through the apparatus for a few seconds. The hydrogen is then turned off slowly, and at the same time the tip of the outlet pipette is sealed in the bunsen flame. The inlet pipette is next withdrawn, and its tip at once sealed.

Lastly, the wide portion of the outlet pipette containing the cartridge is heated moderately in the flame. The apparatus is then ready for the incubator.

To remove the plate culture, it is necessary first to cut off the sealed end of one of the pipettes; the plate can then be easily levered from its bed with the point of a knife. The pipettes if made long enough will last for several cultures. The cultures when removed from the holders may be covered with the tin lids after sterilising them by flaming their inner surfaces.

The whole procedure of cultivation by this method is quite quick and convenient and clean, and unattended with danger *provided there is no gross leakage from defective soldering*. Before use, the plate-holder should be carefully tested for leakage, especially at the sites of attachment of the pipes. This is best done by immersing the holder in water, and blowing through each pipe in turn with the communicating holes blocked with the finger. On one occasion, a gross leakage in one of the pipes resulted in the plate being forced from its bed with explosive violence during the passage of hydrogen.

Below are given notes of culture experiments designed to test the apparatus.

COMPARISON OF SURVIVAL FROM INOCULATION IN PLATE CULTURES AND IN DEEP CULTURES.

In certain experiments carried out with a broth known as P 3, a very high degree of survival from inoculation of certain strains of anaerobes was found to occur in P 3 agar when inoculated as "shake" cultures with seekings of known numbers of organisms (de Smidt, 1923). It was decided, therefore, to test the apparatus, by means of surface plate-cultures and shake-cultures in P 3 agar inoculated in series with as much uniformity as possible; using strains which consistently gave a high yield of colonies in P 3 agar shake-cultures. Uniform inoculation of surface and deep cultures is not easily procurable. An approximation to it was found by inoculation with drops of a saline suspension of spores delivered from a capillary pipette. The pipette was marked some few inches above the end, and after flaming it, a column of the suspension was allowed to flow up to the mark. The sides of the pipette were carefully drained of excess fluid, and one drop allowed to fall upon the surface of the well-dried agar plate. Similar drops of spore suspension were let fall into tubes of uniform diameter containing 10 c.c. of melted agar at 40-45° C. In doing this, the inoculating pipette was introduced into the culture tubes held at an angle, and the drop let fall from close to the surface of the medium. Each tube was then rotated gently 25 times between the palms of the hands before allowing to set. The drop placed on the agar plate was thoroughly spread over the surface with a thin sterile glass rod bent at an angle. A variable number of organisms are lost by sticking to the spreader; in the shake-cultures only a very small proportion of organisms are lost by coming to rest in the oxygenated zone at the surface of the agar column.

Colonies in the shake-cultures were counted when the smallest present had

reached a size of about 1 mm. diameter. The suspensions of spores used were such that one capillary pipette drop yielded considerably less than 100 colonies. Such numbers of colonies are readily counted in a shake-culture tube by marking small quadrilateral areas on its surface with a grease pencil. The colonies in the plate-cultures were counted by similarly marking the surface of the Petri capsule.

The following table gives the colonies counted in plate-cultures and shake-cultures inoculated with *B. sporogenes* of different strains and *B. tetani*.

	Shake-cultures	Hydrogen plate
<i>B. sporogenes</i> ...	18.22	9
	54.67	13
	21.25	18
	27.43	Confluent growth
<i>B. tetani</i> ...	3.1	Filmy growth

In two of the above experiments, the plate was insufficiently dried for discrete colonies.

The next table shows comparisons of the hydrogen plate with a well-known single plate method depending upon alkaline pyrogallate for anaerobiosis. The plates here were inoculated with drops of very light saline suspensions of vegetative organisms from young broth cultures.

	Hydrogen plate	Pyrogallate plate
<i>B. tetani</i> ...	68	Nil
Type 3 c (McIntosh) ...	30	Nil
<i>B. sporogenes</i> ...	69	Nil

The hydrogen plate method has been in use for three years and has given uniformly satisfactory results. Failure of cultures when traced to the apparatus are invariably due to small defects in soldering, resulting in leakage which usually is only discovered by cultural tests.

My thanks are due to Mr C. Ashton, of the Clinical Laboratory, Manchester Royal Infirmary, for his very skilful assistance in photographing the apparatus.

The plate holders were very skilfully made by Mr E. Moulds, Longsight, Manchester.

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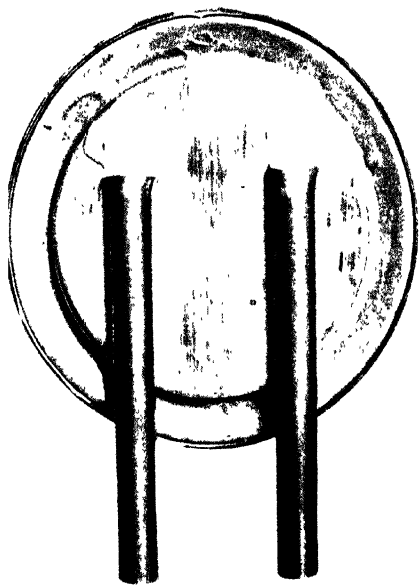


Fig. 1

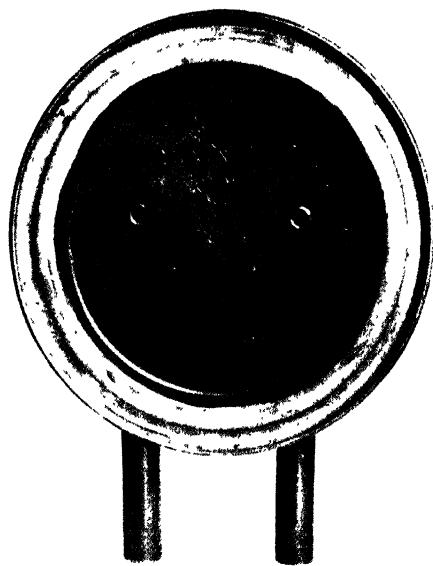


Fig. 2

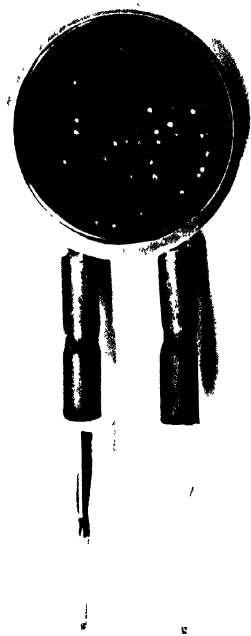


Fig. 3

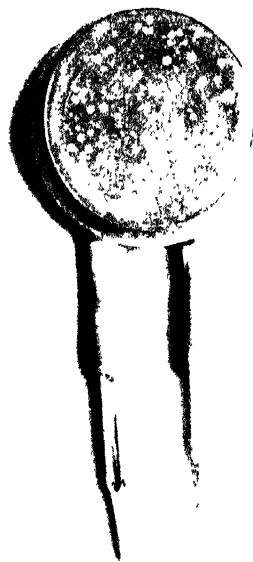


Fig. 4

A FURTHER NOTE ON NATURAL AND EXPERIMENTAL PLAGUE IN TARBAGANS¹.

By WU LIEN-TEH. (G. L. TUCK.)

(From the *Laboratory of the Manchurian Plague Prevention Service, Harbin, China.*)

CONTENTS.

	PAGE
I. A spontaneous outbreak of plague among tarbagans	329
II. Experimental infection of hibernating tarbagans	330
III. The Ecto-parasites of the tarbagan. (a) The ability of the tarbagan flea to bite man; (b) Author's experiments; (c) Seasonal variations in the number of fleas infesting the tarbagan	330
IV. The mode of spread of tarbagan plague and of the associated human infection	332
Conclusion	334

I. A SPONTANEOUS OUTBREAK OF PLAGUE AMONG TARBAGANS.

TOWARDS the end of May, 1923, we received information of two fatal cases of bubonic plague in man, one near Yakoshih (a village on the Chinese Eastern Railway, 168 miles east of Manchouli), and the other at Haranor (in Transbaikalia, 52 miles west of Manchouli); both victims were Russians who had hunted tarbagans and afterwards developed axillary buboes.

At the same time we obtained news of an epizootic among the tarbagans at Sektui, a village in Transbaikalia, 15 miles distant from the Trans-Siberian Railway and 30 miles from Manchouli, the frontier station of Manchuria.

The Russian authorities had established a post in the village with the purpose of investigating the natural occurrence of plague in tarbagans. They chose this site because periodic outbreaks of disease among these rodents are known to occur here, and, indeed, in the summer and autumn of 1921 they were able to obtain definite evidence of plague among the tarbagans.

Dr Pollitzer, Dr Kwan and myself journeyed from Harbin on June 2nd, arrived at Sektui on June 10th, and returned to Harbin on June 23rd. Dr Sukneff, the head of the Russian Plague Detachment, a keen, friendly and experienced investigator, kindly gave us an account of his findings and demonstrated his specimens to us, thereby convincing us that the mortality among the tarbagans in the district was due to plague. On June 10th Dr Sukneff gave us the opportunity of dissecting a tarbagan found on the previous day in the Barun Zasulan valley, 6½ miles south-east of Sektui. The post-mortem findings on this animal were as follows:

Autopsy on Tarbagan: Large superficial bubo on left side of neck; salivary glands oedematous and congested; cervical glands on left side much enlarged, showing caseous and

¹ Abstract of MS. received from Author 11. ix. 1923.—Ed.

330 *Natural and Experimental Plague in Tarbagans*

purulent matter on section. Glands on right side of neck swollen but not purulent. Spleen enlarged and soft. Liver swollen and congested. Some clear liquid in pericardial sac. Both lungs showed congested areas, especially the right lung almost the whole lower lobe of which was affected. *B. pestis* present in smears of all organs. Cultures and inoculation experiments on guinea-pigs gave positive results for plague.

Summary of results of Histological Examination:

(a) *Cervical bubo*: A portion of the caseating area was cut. There was marked leucocytic infiltration adjoining the caseating region; *B. pestis* in isolated masses found in and around malpighian bodies; haemorrhages in lymph spaces; capsule of gland infiltrated. (b) *Sub-maxillary Salivary gland*: Intense infiltration; haemorrhages into and around acini; *B. pestis* in small numbers among lobules; epithelial cells of acini mostly show granular degeneration; lymph spaces invaded by red blood corpuscles. (c) *Lungs*: A portion of congested area of right lung was cut. It showed marked early pneumonia; most alveoli invaded by red corpuscles and leucocytes; some alveoli collapsed. Many *B. pestis* in alveoli and interalveolar spaces; proliferation of cells of infundibulae; intense reaction. (d) *Spleen*: Lymph nodules darkly stained; *B. pestis* in great numbers in and between malpighian bodies; capsule infiltrated; blood vessels dilated; lymph spaces invaded by red corpuscles.

Note. On June 23rd, after our departure, a decomposed tarbagan was found in the same valley. A guinea-pig inoculated with material from this tarbagan died of typical plague.

We brought back to Harbin four strains of *B. pestis* which had been isolated from three tarbagans by the Russian Commission. These strains conformed to the usual cultural tests for *B. pestis*, and killed guinea-pigs with typical signs of acute plague, when rubbed in the skin.

II. EXPERIMENTAL INFECTION OF HIBERNATING TARBAGANS.

Zabolotny and Tchurilina (1911) using tarbagans, and Dujardin-Beaumetz and Mosny (1912) using European marmots, found that when these animals were inoculated with plague while in the hibernating state, the infection ran an unusually prolonged course before death ensued.

During the winter 1922-1923 we exposed to infection six hibernating tarbagans, three by spraying, two by way of the nostrils, and one by the cutaneous method. The illness lasted from seven to eighty-six days, and all the animals died. Two showed the signs of acute plague; in two the appearances suggested those of "resolving plague"; and two showed no definite evidence of plague.

III. THE ECTO-PARASITES OF THE TARBAGAN.

In a recent paper Jettmar (1923) gives a detailed list of the ecto-parasites of the tarbagan; these include the tarbagan flea, *Ceratophyllus silantiewi* Wagner (Wagner, 1898), lice (*Polyplax*) and ticks (*Rhipicephalus*).

(a) *The ability of the tarbagan flea to bite man.*

Petrie (1911) found that this flea is able to bite man. In a supplementary note (1912) he states that a description of it is given by Tiraboschi (1903-1904), who recognised its importance as a possible carrier of plague infection; and that Dudchenko (1909) noted that "in the fur of the tarbagan there are always numerous fleas with a long body and reddish-brown colour. . . . The fleas do not

Table I. *Winter Experiments 1922-23.*

No. of tarbagan	Date of infection	Mode of infection	Condition during experiment	Date of death	Days sick	Autopsy result
T. 197	9. xi.	Inhaled	Continuously feverish	16. xii.	37	Chronic plague
T. 223	27. xi. 14. xii.	„	Hibernating	25. ii.	59	Smears suspicious, but cultures sterile
T. 233	21. ii.	„	„	14. iii.	21	Acute plague
T. 238	21. ii.	„	„	3. iv.	41	Resolving plague (?)
T. 231	8. iii.	„	Feverish	13. iii.	5	Acute plague
T. 232	18. iii.	„	„	14. iii.	6	„ „
T. 224	19. ii.	Nostrils	Hibernating	26. ii.	7	„ „
T. 250	1. iii.	„	„	26. v.	86	Negative for plague
T. 241	30. i.	Cutan.	„	14. iv.	74	Resolving plague (?)
T. 236	12. iii.	Conj.	Slight local reaction, feverish	29. iii.	17	Subacute plague
T. 251	2. iii.	„	Sick, not hibernating	28. v.	157	No signs of plague
T. 213	11. xi.	Contact	Hibernating	12. i.	62	No signs of plague. Fed Corpse 197 16. xii.
T. 234	26. xi.-14. iii.	Contact from fed Corpse 232	Hibernating	19. iii.	113	Acute plague (intestine affected?)

live on man, but, if opportunity offers, they bite him. The bites produce red spots and sometimes swellings resembling urticaria." Some months later, in 1911, the author (W. L. T.) proved in a number of experiments that *Ceratophyllus silantiewi*, when hungry, readily fed upon man. Dudchenko (1915) mentions an instance where, in the process of digging up a tarbagan burrow, one of his workmen was bitten by a flea, which proved to be the specific flea of the tarbagan. Jettmar (1923) also notes the ability of this flea to imbibe human blood.

(b) *Author's Experiments.*

The following record relates to recent experiments of ours with *C. silantiewi*:

23. v. 1923: Two fleas (*a*) and (*b*) which had been starved for four days were used. Flea (*a*) bit a man, remaining attached to his skin for 31 minutes; flea (*b*) refused to bite.

25. v. 23: Flea (*a*) again bit a man for ten minutes.

26. v. 23: Flea (*a*) bit a healthy guinea-pig for ten minutes.

31. v. 23: Flea (*c*) was allowed to bite a plague-infected guinea-pig for ten minutes. Next day it was fed on a healthy tarbagan and a healthy guinea-pig, but both animals remained well. This flea survived for 18 days, when, after its death, it was ground up in saline solution; the emulsion was injected intraperitoneally into a guinea-pig, but no plague resulted.

Later experiments showed the periods of attachment of tarbagan fleas to the skin of human beings to be 13, $4\frac{1}{2}$, 3, and 2 minutes respectively. We have seen only on one occasion a flea deposit its faeces on the skin, but never while biting.

We may add here that ticks (over 30) were repeatedly given the chance but refused to bite; a louse bit a man for 5 minutes.

(c) Seasonal variations in the numbers of fleas infesting the tarbagan.

The available data are as follows: (1) Petrie at the time of the Mukden Conference (April, 1911) reported that the numbers of fleas on twelve tarbagans (brought from Manchouli to Mukden, a distance of 925 miles) were 2, 2, 2, 3, 0, 2, 2, 5, 2, 0, 12, 3, respectively, an average of 3 fleas per tarbagan. (2) The author (W. L.-T.) examined a number of freshly caught tarbagans from August to September, 1911, in Mongolia, and found the flea-rate to vary within wide limits. On one animal 94 fleas were caught, and the number on each was seldom less than 5; the average was over 10. Occasional observations made by Russian medical men (Wassilewski and others) also suggest a high flea-rate in the autumn. (3) Our flea counts in May—June, 1923, were as follows:

At Manchouli. May 19th: 20, 2, 0, 1, 0, 1, 0, 0 fleas on 8 animals examined.

At Harbin. May 29th: 0 fleas on 14 animals freshly arrived from Manchouli (584 miles).

At Manchouli. June 6th. Two tarbagans arrived on the previous evening and were kept in a tin bucket. Next morning one was found dead; 4 fleas, 6 ticks and 4 lice were taken from it. The surviving animal had no fleas.

At Manchouli. June 6th: 0, 0, 1, 0, 0 fleas on 5 animals examined.

 " " 7th: 0, 3, 1 " " 3 "

 " " 9th: 0, 0, 0, 0, 0, 1, 0, 2 " " 8 "

The figures indicate that the flea-average in spring is small, when compared with that in autumn, and in this respect are significant because human plague is generally reported from Siberia towards the end of summer or in the autumn, that is, during the season for hunting the tarbagan and during the harvest, when human beings come into contact with this animal.

IV. THE MODE OF SPREAD OF TARBAGAN PLAGUE AND OF THE ASSOCIATED HUMAN INFECTION.

The mode of spread of plague-infection among tarbagans: Strong, the chief delegate of America to the International Plague Conference, read a paper in April, 1911, which showed that the tarbagan is susceptible to acute plague; and Zabolotny and Tchurilina two months later proved the existence of spontaneous plague infection in a tarbagan.

The Russian investigators have since obtained ample proof of epizootic plague in tarbagans and of associated human infections. Petrie (iv. 1911) gave a brief account of the flea infestation of the tarbagan and pointed out the significance of his observations, when considered in the light of knowledge of the transmission of plague amongst rats by means of the rat flea. We have attempted a number of experiments with fleas, lice and ticks from tarbagans, but have up to now failed to obtain a positive result. One difficulty in conducting such experiments is that the tarbagan is a strong vicious animal, not easily handled; its mode of life in wild remote districts is an added difficulty.

The number of cases of natural infection that we have been able to collect from various sources is small (13), but they include examples of cervical, inguinal, and axillary buboes, a distribution that is consistent with a skin infection caused by plague-infected fleas. Although the tarbagan is by nature herbivorous, we have often seen it devouring the carcass of its mates, when kept in confinement. But this means of transmission, if it occurs naturally, must be very rare.

Table II. *Tarbagans with Natural Plague.*

No.	Date found	Locality	Remarks
1	Autumn 1907	Between Lake Dalainor and Manchouli	No buboes, lungs free. Suspicious chronic plague.
2	17. vi. 1911	Lake Charbada	Decomposed. Right axillary bubo. Smears and cultures positive. Expts. negative.
3	24. vi. 1911	Sharasun	Cervical buboes. Haemorrhage in lungs. Expt. on tarbagan positive.
4	26. vi. 1911	Arabulak	No buboes, lungs free. Expt. upon mice positive.
5	16. ix. 1921	Kinkija (5 v. from Sektui)	Cervical and axillary buboes. Haemorrhage lungs. Guinea-pig expt. positive.
6	19. ix. 1921	"	Inguinal buboes. Otherwise as under (5).
7	ix. 1921	"	Remnants only. Smears from muscle positive.
8	22. ix. 1921	Barun Zasulan (10 v. from Sektui)	Cervical bubo. Smears positive.
9	29. iv. 1923	Chistokina (1½ v. from Sektui)	No buboes. Lungs congested. Smears positive. Cultures and expts. negative. Dissected two days after being found.
10	4. vi. 1923	Barun Zasulan	Decomposed. Left cervical bubo. All tests positive.
11	8. vi. 1923	"	No buboes. Haemorrhagic foci in lungs. Nodes in spleen. All tests positive.
12	9. vi. 1923	"	Cervical buboes. Haemorrhagic foci in lungs. All tests positive.
13	23. vi. 1923	"	Decomposed. Left axillary and left cervical buboes. Pneumonic areas in lungs. Guinea-pig expt. positive.
14	4. vii. 1923	Substation 82	Remnants only. Smears from muscle positive.
15	21. vii. 1923	Sun Zasulan	" " "
16	25. vii. 1923	Barun Zasulan	Complete specimen. Harbin museum. Cervical buboes. Pneumonic areas in lungs. All tests positive.

Table II shows that the naturally infected tarbagans were mostly confined to an area around Sektui. The plague detachment worked about this district because of its regular periodic outbreaks. It is possible that other foci might have been located if the same attention had been paid to them, but difficulties of communication and transportation have to be considered.

The mode of conveyance of the plague bacillus from the tarbagan to man: Petrie (iv. 1911) expressed the view that the ability of the tarbagan flea to bite man helped to explain the mode of transference of the infection from the tarbagan to man in bubonic cases. He also noted the possibility that a bubonic or septicaemic case in man originating in this way, might, if a secondary plague pneumonia supervened, give rise to a case of primary pneumonic plague. We think that the risk of transference of the infection by the means of the tarbagan flea is easily conceivable amongst those who hunt this animal or use it as food; but, obviously, direct evidence on this point is difficult to obtain. A concurrent

334 *Natural and Experimental Plague in Tarbagans*

risk is that abrasions or small wounds of the hands may become infected as the result of handling infected tarbagans. In a number of instances the infection has been attributed to eating tarbagan flesh, but there seems to be no real foundation for this supposition, and, having regard to the generally accepted view that primary intestinal plague in man is rare or non-existent, it is necessary to receive such reports with scepticism, if unsupported by precise data.

CONCLUSION.

The Delegates to the International Plague Conference¹, which was held in Mukden in April, 1911, at the invitation of the Chinese Government, reached the conclusion, from the evidence presented to them, that "there is strong presumption for believing that tarbagan disease is closely associated with pneumonic plague in Manchuria, Transbaikalia, and north-east Mongolia, and, therefore, with the recent outbreak" (1910-1911).

We now consider it as practically certain that the tarbagan gives rise to the outbreaks of plague which occur almost yearly in Transbaikalia and that it started the outbreaks in Manchuria in 1910-1911 and 1920-1921.

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¹ The signatories of the report of the delegates to the Chinese Government are: Strong, U.S. of America; Worell, Austria-Hungary; Broquet, France; Martini, Germany; Petrie, Great Britain (with the late Dr R. Farrar and Dr D. G. Gray); Galeotti, Italy; Kitasato, Japan; Gonzalez-Fabela, Mexico; Hehewerth, Netherlands; Zabolotny, Russia; and Wu Lien Teh, China.

A COMPARATIVE BACTERIOLOGICAL STUDY OF BOVINE ABORTION AND UNDULANT FEVER.

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PART II.

IN a previous communication on this subject (Khaled, 1922) the most important conclusions arrived at were these:

1. *Brucella abortus* Bang and *B. melitensis* Bruce, are morphologically identical and cannot be differentiated by cultural, biochemical or staining methods, nor by the agglutination reaction.
2. From absorption-agglutination experiments, *B. melitensis* appears to be a substrain of *B. abortus*.
3. Dose for dose, *B. abortus* is much less virulent for guinea-pigs (injected intraperitoneally) than *B. melitensis*.
4. One monkey vaccinated intravenously with killed suspensions of *B. abortus* was found to have become immune to a subsequent infection with living *B. melitensis* whilst the control monkey showed signs of the disease.

Repetition of cross-immunisation experiment. The importance of the cross-immunisation experiment made it necessary to have it repeated with a larger series of monkeys. Four *Macacus* monkeys were chosen; three for vaccination and infection, and one for infection only (to act as control). The following is a typical experiment:

(1) *Macacus rhesus* "A" ♀, received the following inoculations:

3. ii. 22. 1/4 slope of killed *B. abortus*, strain "900," suspended in 2 c.c. saline.

12. ii. 22. 1/2 slope of same in 2 c.c. saline.

20. ii. 22. Do.

Serum tested on 28. ii. 22 agglutinated *B. abortus* in 1 : 3200 and *B. melitensis* in 1 : 800.

(2) *Macacus rhesus* "B" ♂, not previously immunised, to act as control.

Final test. Both monkeys were inoculated on March 3rd with 1/2 slope (intravenously) of living *B. melitensis* "893."

After 18 hours *B. melitensis* was recovered from both monkeys, but at the end of the first week it was possible to recover it only from the control and not from the blood of the immunised monkey.

On the eighth day the titre of the serum of "A" was 1 : 6400 for *B. abortus* and 1 : 1600 for *B. melitensis*, while the serum of "B" agglutinated *B. abortus* in 1 in 100 and *B. melitensis* in 1 in 200 only.

From the tenth day onwards signs of illness (emaciation, weakness, irregular undulating fever and diminished appetite) showed themselves in the control but not in the immunised monkey. In this the only effect of injecting living *B. melitensis* was to cause a rise in the agglutination-titre of its serum (for *B. melitensis* and *B. abortus*) and an initial loss of weight which was soon regained. The following table records the weights of the two monkeys so that they may be compared:

	<i>M. rhesus</i> "A"	<i>M. rhesus</i> "B" (control)
Before infection	3800 gm.	3700 gm.
10. iii. 22	3700	3590
15. iii. 22	3650	3540
22. iii. 22	3610	3500
30. iii. 22	3630	3460
6. iv. 22	3690	3430
24. iv. 22	3750	3400
4. v. 22	3790	3360

Post-mortem examination. On June 4th, i.e. about three months after infection, both animals were killed. No naked-eye changes were seen except enlargement and congestion of the spleen of "B" (control). From this as well as from the heart's blood a pure culture of *B. melitensis* was recovered.

No organisms could be recovered from the tissues of monkey "A" (immunised).

The same procedure was adopted in immunising and infecting the other two monkeys with the exception that other strains of *B. abortus* were used for vaccination. The same results were also obtained except that in one monkey there was no increase in the agglutination-titre of its serum after infection with *B. melitensis*.

From this it seems that emulsions of killed *B. abortus* inoculated intravenously can protect monkeys against a subsequent infection with living *B. melitensis*.

CROSS-IMMUNISATION OF GOATS.

Vincent (1918), by using ether-killed polyvalent vaccine (*B. melitensis* and *B. paramelitensis*), was able to immunise a series of goats sufficiently to withstand massive doses of the living virus given subcutaneously, intravenously and intraperitoneally. No germs could be recovered from milk or post-mortem.

Marich, Sultana and Mifsud (1921), working under Prof. Zammitt, tried to repeat Vincent's experiments on the Island of Malta, but they failed to corroborate his results, getting practically as many infections in the "immunised" goats as in the control animals. On reading the papers of these authors one is struck by the circumstance that the toxicity of *B. melitensis* necessitated the use of moderate doses in immunisation and that the resulting

immunity was therefore very small as judged by the low agglutination-titre and by the fact that the immunised animals contracted the disease. Burnet (1923) also failed to immunise goats against *B. melitensis* by vaccinating the animals with the killed organism.

In 1921 the writer inoculated two goats with a single slope of living *B. abortus* and found that the agglutination response to both *B. melitensis* and *B. abortus* was very distinct; yet because the goats were not in milk at the time, it was not thought of much use trying to infect them with living *B. melitensis*. The success of the monkey cross-immunisation experiments detailed above and in a previous communication suggested that it would be well worth while to see if it could be applied to goats, *i.e.* to see whether immunisation with living *B. abortus* would prevent the excretion of *B. melitensis* in milk after infection of the animal with this latter organism.

TECHNIQUE OF EXPERIMENT.

A. Milk:

1. Collection:

The udders were first cleaned with soap and water, then with lysol solution (1 in 1000) and afterwards with alcohol, special care being paid to the nipples. When dry, they were milked with clean, sterile-gloved hands. The first few cubic centimetres of milk (from each nipple) were rejected. The rest was received in sterile Erlenmeyer flasks, which were plugged when full and marked with the number of the goat and the date and hour of the milking. By using the above precautions very few extraneous organisms could find their way into the milk.

2. Culture:

Two methods were used in parallel so as to obtain more trustworthy results:

(a) *Direct plating*: 1–2 c.c. of the milk, drawn with a sterile pipette, were allowed to spread on a glucose-agar plate. After the milk film had dried the plates were inverted and incubated at 37° C. Examination for suspicious colonies was made after one, two, three, and five days.

(b) *Indirect plating*: 0.5, 1, and 2 c.c. of milk were added, each to a glucose-broth tube containing 10 c.c. of media. After shaking, the tubes were incubated for three days and loopfuls were plated out each day and the plates examined for suspicious colonies. This method apparently favours the multiplication of *B. melitensis*, the goat's milk seemingly acting as a favourable adjunct to the media and thus increasing the chances of detecting the organism if the initial infection of the milk is small.

3. Agglutination:

Whey was used in preference to whole milk as a sharper end point could be obtained. To 10–15 c.c. of the milk, a few drops of acetic acid were added and the milk allowed to stand. The separated whey was filtered off and the filtration repeated until a limpid fluid was obtained. This was neutralised with

N/10 NaOH. The neutral fluid was put up for agglutination, dilutions of 1 : 20 to 1 : 1280 being used.

B. Blood:

1. Culture:

The neck was shaved in the region of the external jugular vein. After painting the space with tinct. iodi., 10 c.c. of blood are drawn aseptically and placed in 100 c.c. of glucose broth in an Erlenmeyer flask and incubated. Glucose agar plates were inoculated and the plating and incubation continued for seven days.

2. Agglutination:

The agglutination was carried out in small tubes 5 cm. long by 0.5 cm. diameter. The serum dilution was made up with normal sterile saline in series 1 : 50 to 1 : 12,800, while the bacterial emulsion was made up in sterile distilled water to which formalin (1 : 1000) was added. After two hours' incubation at 37° C., the tubes were placed at room temperature and read next day (*i.e.* 18 hours later). Positive tubes usually showed sedimentation.

The two goats selected for cross-immunisation were healthy looking and were giving a very good output of rich milk. They had kidded 12 and 21 days previously.

Samples of their blood and milk were cultured for *B. melitensis* (and *B. abortus*) and their sera and whey tested for *B. melitensis* and *B. abortus* agglutinins. All these tests were negative.

Immunisation was started on February 23rd, 1923, each goat receiving intravenously the following doses:

Date: day
and month

Feb. 23 One slope of *B. abortus* "900" (72 hours old growth).

„ 28 Three slopes *B. abortus* "900" emulsified in 10 c.c. saline.

Mar. 5 Six slopes *B. abortus* "900" in 15 c.c. saline.

Final test „ 12 *Infected intravenously* with three slopes of *B. melitensis* "893," 72 hours old, emulsified in 10 c.c. saline.

The response of the two goats to these inoculations was as follows (Table I):

Throughout the experiment the animals were quite happy and partook freely of their food, but the output of milk gradually diminished until at the end it was reduced to about 150 c.c. per diem. No local signs or symptoms of inflammation of the udders or adnexa were noticeable.

Table I.

Date 1923: day and month	Blood culture		Milk culture		Serum agglut.		Whey agglut.	
	Goats I and II		Goat I	Goat II	Goat I	Goat II	Goat I	Goat II
Feb. 24	<i>B. abortus</i>		Negative		Negative		Negative	
27	Negative		<i>B. abort.</i> Neg.		<i>v. ab.</i> 1 : 100 <i>v. me.</i> 1 : 50	Neg.	"	
Mar. 3	"		Neg. <i>B. abort.</i>		<i>v. ab.</i> 1 : 800 <i>v. me.</i> 1 : 400	1 : 400 1 : 200	<i>v. ab.</i> 1 : 40 <i>v. me.</i> 1 : 20	Neg.
4	"		Negative		<i>v. ab.</i> 1 : 1600 <i>v. me.</i> 1 : 800	1 : 800 1 : 400	<i>v. ab.</i> 1 : 40 <i>v. me.</i> 1 : 20	1 : 20
7	"		"		<i>v. ab.</i> 1 : 800 <i>v. me.</i> 1 : 3200	1 : 400 1 : 1600	<i>v. me.</i> 1 : 20 <i>v. ab.</i> 1 : 80	1 : 20 1 : 40
8	"		"		<i>v. me.</i> 1 : 1600	1 : 800	<i>v. me.</i> 1 : 80	1 : 40
10	"		"		Same		Same	
12	"		"		<i>v. ab.</i> 1 : 6400 <i>v. me.</i> 1 : 3200	Same Same	<i>v. ab.</i> 1 : 160 <i>v. me.</i> 1 : 80	1 : 80 1 : 40
(Inoculation with <i>B. melitensis</i>)					<i>v. ab.</i> 1 : 12800 <i>v. me.</i> 1 : 6400	Same	<i>v. ab.</i> 1 : 320 <i>v. me.</i> 1 : 160	1 : 160 1 : 80
13	<i>B. melitensis</i>		"		Same		Same	
15	Negative		"					
18	"		"		<i>v. ab.</i> 1 : 12800 <i>v. me.</i> 1 : 12800	—	<i>v. ab.</i> 1 : 320 <i>v. me.</i> 1 : 160	1 : 160 1 : 80
24	"		"		Same		Same	
26								
27								
28								
29								
30								
Apr. 2								
3	—		—		—		<i>v. ab.</i> 1 : 160 <i>v. me.</i> 1 : 160	1 : 160 1 : 80
4	Negative		Negative		<i>v. ab.</i> 1 : 12800 <i>v. me.</i> 1 : 12800	1 : 12800 1 : 6400	—	
5-13	"		"		Same		Same	

CONTROL GOAT.

A healthy well-fed goat that had kidded four days previously, giving daily about two pints of very rich milk.

The blood and milk were cultured for *B. melitensis* and the serum and whey tested for *B. melitensis* and *B. abortus* agglutinins. All these tests gave a negative result.

On March 15th it was infected intravenously with three slopes of *B. melitensis* "893" (72 hours old), emulsified in 10 c.c. sterile saline. Table II shows the response to the inoculation.

The output of milk diminished slightly but the most marked phenomenon was the swollen, red, hot and tender state of the udders whilst the general condition showed apparent depression, inactivity and emaciation.

Post-mortem examination. On April 13th, the three goats were anaesthetised by chloroform, after tying their legs. They were then bled to death.

In the two immunised goats no distinct naked-eye change was noticed in the spleen, liver or the mammary gland, nor any microscopic change detected in these organs or in the sections of one of the deep glands. Cultures taken from all these tissues and from the heart's blood were negative.

In the control goat the noteworthy change was the acute inflammatory condition of the mamma and the enlargement of the spleen. Cultures from

the mamma, spleen and heart's blood gave a growth of *B. melitensis*, those from the liver and a deep lymph gland were negative.

The number of experimental animals is small but the results as seen in Tables I and II are marked. *B. abortus* appeared in the milk during immunisation only once or twice and then died out and was eliminated; a high degree of immunity was obtained and no clinical signs followed an intravenous dose of three slopes of living *B. melitensis*, nor could anything be recovered from repeated blood and milk cultures nor from the spleen or liver after death.

Table II.

Date 1923: day and month	Blood culture	Milk culture	Serum agglutination		Whey agglutination	
			v. <i>B. abortus</i>	v. <i>B. melitensis</i>	v. <i>B. abortus</i>	v. <i>B. melitensis</i>
Mar. 15 (Infected with <i>B. melitensis</i>)	—	—				
16	<i>B. melitensis</i>	Negative		Nil		Nil
18	"	"	1 : 100	1 : 100		"
19	"	"	1 : 200	1 : 200		"
20	"	"		Nil		"
21	"	"		"		"
22	"	"	1 : 400	1 : 800		"
23	—	"		Nil		"
24	—	<i>B. melitensis</i>	1 : 800	1 : 1600		"
25	—	"				
26	—	"				
27	—	"				
28	—	"				
29	<i>B. melitensis</i>	Negative	1 : 1600	1 : 6400	1 : 20	1 : 40
30	—	"				
31	—	Positive				
Apr. 2	—	"	—	—	1 : 80	1 : 80
3	—	"				
4	—	"	1 : 1600	1 : 6400	1 : 80	1 : 160
5-12	<i>B. melitensis</i>	"	Same	Same	Same	Same

In the control goat, on the other hand, an acute mammitis set in and the organism was recovered repeatedly from the blood and milk during life and from the spleen at autopsy.

The results of this experiment are comparable with the finding of Pratt-Johnson (1921) in the production of immunity against *B. cholerae suis*. He showed the possibility of securing solid immunity against a highly virulent organism by vaccination with living members of a related type of low virulence rather than with the killed antigen of a homologous strain.

ATTEMPTS TO APPLY THESE EXPERIMENTAL RESULTS TO THE TREATMENT OF UNDULANT FEVER IN MAN.

A survey of recorded attempts to treat undulant fever with *B. melitensis* antigen reveals several common features, whether the antigen used has been of the ordinary or sensitised type or whether it has been autogenous or stock:

- (1) Very small doses, 50-200 millions, have to be used.
- (2) Long intervals of ten days have to intervene between the injections.
- (3) The vaccine should be used only when the temperature has fallen or is falling.

The vaccine is not indicated in the acute cases owing to the great toxicity of the virus.

What appears to be desirable therefore in a disease like undulant fever is a vaccine of such low virulence as will permit its usage in all cases, acute and mild, at any period of the disease, at frequent intervals and in such doses (if possible living) as will produce sufficient immunity to cut down the fever. It was therefore thought worth while to test whether *B. abortus* vaccine might satisfy these requirements. Three cases of undulant fever have been treated in this way.

Case I. M. H., 35 years old, suffering from a severe fever, the temperature reaching 104° F. in the evening and little less in the morning. Widal test carried out on the 40th day of the disease was negative for *typhoid* and *paratyphoid* but positive for *melitensis*; the serum agglutinating the laboratory strain up to 1 : 800. Blood culture on the 45th day of the disease gave a pure *B. melitensis* growth. The condition of the patient being rather bad, it was not felt advisable to treat him with *melitensis* vaccine. The physician, therefore, agreed to try the much less toxic, killed, *abortus* vaccine. A first dose of 300 millions was given. This produced no ill-effect and was followed, five days later, by a second of 600 millions. The subsequent doses were 800, 800, 1000, 1500 and 2000 millions at five-day intervals. The temperature began to show a drop after the fifth injection and came down to normal two days after the eighth injection, *i.e.* 37 days from starting the treatment.

Case II. W. H., 27 years old. Blood sent for culture on the tenth day of the fever, on suspicion of enterica. Culture gave pure growth of *B. melitensis*. Serum agglutinated laboratory *melitensis* strain in 1 : 200 dilution. A dose of 500 millions of killed *B. abortus* was given subcutaneously on the 16th day of the disease. Five days later a second dose of 500 millions was given, followed by 800, 800, 1000 and 1500 millions at five-day intervals. The body reaction was bearable. The case could not be classified as an acute, severe one. The temperature became normal 26 days from the commencement of vaccine treatment.

Case III. H. A., 30 years old, suffering from fever for five months, with occasional afebrile periods. His serum agglutinated *B. melitensis* in 1 : 1600 dilution. Blood examined culturally on three weekly occasions gave a negative result. The fever was rather mild, but the emaciation was marked, sweating intense and joint pains severe. The initial dose given was 600 millions. There being not much reaction, a second injection of 800 millions was given after five days and repeated after another five days. These three doses did not have much effect on the temperature but the patient began to feel a general well-being, the joint pains ameliorated a little, and the sweating definitely diminished. Subsequent doses of 1000, 1500, 1500 and 2000 millions were given. The last dose was repeated three times before the temperature dropped to normal. With each dose the general condition of the patient got better and his weight increased.

As seen from these three cases higher doses of *abortus* vaccine could be used in the treatment than is possible with *melitensis* vaccine. The temperature charts (not here detailed) show curtailment of the course of the fever, especially in Case II, whilst in all three cases the patients felt and looked better under the vaccine treatment. The number of cases, however, is very small and the method requires repetition before a definite conclusion can be formed¹.

SUMMARY.

1. Certain cross-immunisation experiments (on monkeys) with *B. abortus* and *B. melitensis* carried out in 1921 were repeated with the same effective result.

2. It was found that goats vaccinated intravenously with *B. abortus* in massive doses are protected from subsequent infection with a virulent *B. melitensis* strain, and fail to pass that organism in the milk.

3. The record of the effect of *B. abortus* vaccine in treating three cases of undulant fever is given.

In conclusion I wish to express my thanks and gratitude to the staff of the Lister Institute for the hospitality of the laboratories and for many valuable criticisms.

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¹ Nicolle, Burnet and Conseil (1923) have also recently shown that living *B. abortus* may be given with impunity to healthy subjects (five tested). 800–900 millions of the living 24 hr. culture were given subcutaneously without causing fever or other troublesome symptoms and they also suggest the possibility of employing *B. abortus* in the prophylaxis and vaccine-therapy of undulant fever. *Compt. rend. Acad. Sci.* CLXXVI. 1034. [Editor's note.]

A SMALL OUTBREAK OF DYSENTERY ASSOCIATED WITH AN UNUSUAL BACILLUS.

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IN January, 1923, a small outbreak of dysentery occurred amongst the nursing staff of a certain institution. In all there were six cases. Only one case, the first which occurred, showed symptoms of any great severity. In the remaining five cases the symptoms were slight, and the patients were able to return to duty in seven or eight days from the commencement of the illness.

In each of these five cases the onset was marked by fever, malaise, pain in the abdomen, frequency of motions and some tenesmus. For the first 48 hours or so the temperature showed elevation to 102° F. or 103° F., but subsequently quickly returned to normal. At the commencement of the disease there were, on the average, five to eight motions in the 24 hours, but in two or three days this frequency ceased and the number became normal. Blood and mucus or mucus alone were present in the faeces in the early stages.

The symptoms in the first case were of greater severity. The patient had a more protracted convalescence, and did not return to duty until more than a month had elapsed. There was fever for the first week. For the first three or four days the number of stools per 24 hours was six to eight and blood and mucus was present. After this period the frequency gradually abated and the blood and mucus almost disappeared from the faeces, but there was some recurrence of this symptom during the early part of the second week, although at this period the temperature had returned to normal. The abdominal pain, a diffuse pain over the lower part of the abdomen, was a prominent feature in this case and with the tenesmus lasted considerably longer than in any of the succeeding five cases.

Reviewing the symptomatology as a whole I think that this outbreak of dysentery would appear to have shown a distinct resemblance in severity to the milder cases of Flexner dysentery, as seen in Eastern countries where dysentery occurs in epidemic form.

An investigation into the source of infection revealed nothing. The cases did not occur simultaneously, but followed one another at intervals of a few days. All the nurses who contracted the disease took their meals in the same room, but all had been working in different wards. None had been employed in the nursing of sick children.

BACTERIOLOGICAL EXAMINATION.

Specimens of faeces were examined from four cases, namely from the first case with severe symptoms (No. 1) and from three of the remaining cases (Nos. 2, 3 and 4). From three of the cases, Nos. 1, 2 and 3, a similar organism was obtained.

The faeces were plated on litmus lactose agar. The lactose used was chemically pure and was unaffected by organisms of the typhoid, paratyphoid, and dysentery (Shiga and Flexner) groups.

The first specimen from case No. 1 was obtained on the third day of the disease. Blood and mucus were abundant and an almost pure culture of the organism was obtained. A second specimen ten days later was examined with negative result.

Specimens of faeces from No. 2 (blood and mucus present) and from No. 3 (mucus present, but no blood macroscopically) were also obtained early in the disease. About a dozen colonies of the organism were found in No. 2, and a large number of colonies in No. 3 on the plates at the end of 24 hours' incubation.

Two specimens of faeces were received from No. 4 at an early stage of the infection, but examination gave negative results.

The organism obtained from the first three cases was found to be a Gram negative non-motile bacillus, showing the size and shape and the long involution forms seen in many bacilli of the coli-typhoid group. The colonies on litmus lactose agar at the end of 24 hours were definitely blue and their edges were round. They were much larger than colonies of the Shiga and Flexner dysentery bacilli. At the end of 48 hours a further increase in size had occurred: the edges were becoming uneven and the blue colour intensified in their centres. The reactions of all three on carbohydrates, using 1 per cent. carbohydrate with 1 per cent. peptone, and a Lemco broth basis and 1 per cent. of 0.06 phenol red as indicator, with slight variations were similar. Acid was formed in glucose, mannite and maltose at the end of 24 hours and remained constant for a period of ten days. In saccharose acid was formed about the fourth day and in lactose about the seventh day. Though a slight preliminary acidity might occur in dulcitate, this medium became definitely alkaline on the third day, a reaction which was increased at the end of ten days' incubation. Inulin and salicin were also rendered distinctly alkaline on the fourth day with an increased reaction later. No gas was formed in any of these media. Milk became acid at the end of 24 hours and more acid in 48 hours. This acidity remained unchanged. In the case of No. 1, slight clotting was observed on the ninth day, and was complete on the tenth day. No. 2 showed partial clotting on the twelfth day and complete on the thirteenth day. No. 3 showed slight clotting on the tenth day and complete clotting on the eleventh day. Examined after ten days' incubation in 1 per cent. peptone all three organisms showed only a trace of indol formation. They were non-haemolytic and produced no liquefaction of gelatin after fourteen days.

No effects were obtained by feeding a rabbit with living cultures of the bacillus. Pieces of carrot soaked with an emulsion were given daily for one week. The animal showed no symptoms and there was no loss of weight. An examination of the serum at the end of that time failed to reveal the presence of any specific agglutinin.

Rabbits were also inoculated with increasing doses of 24 hour live agar cultures of organisms Nos. 1 and 2. No symptoms and no loss of weight were observed to occur in the animals during the period of these injections.

In the case of No. 1 six injections were given at intervals of five or six days commencing with a dose of 100 millions and concluding with 1500 millions. In testing the serum for agglutinins it was found best to employ live agar emulsions. Suspensions in Dreyer's veal broth medium, both living and formalised, did not give such satisfactory results. After this series of injections the serum of this animal was found to agglutinate all three organisms in a dilution of 1/2000.

In the case of No. 2 three injections, 250, 500 and 1000 millions were given, and the serum obtained agglutinated all three organisms in a dilution of 1/1000.

A slight difference was found, however, by absorption experiments and this is illustrated in the following tables:

Table A.

Rabbit anti-serum prepared by immunisation with bacillus No. 1.

End point of untreated serum	End point after saturation with bacillus No. 1	End point after saturation with bacillus No. 2
On No. 1—1 in 2000	Nil	Nil
On No. 2—1 in 2000	Nil	Nil

Table B.

Rabbit anti-serum prepared by immunisation with bacillus No. 2.

End point of untreated serum	End point after saturation with bacillus No. 1	End point after saturation with bacillus No. 2
On No. 1—1 in 1000	Nil	Nil
On No. 2—1 in 1000	1 in 200	Nil

It will be seen that saturation of anti-serum No. 2 with organism No. 1 failed to remove completely all agglutinins for organism No. 2.

The rabbit anti-serum No. 1 was also tested against Typhoid, Shiga and Flexner antigens. In the case of Flexner two strains were used. At the end of five hours' incubation no agglutination was obtained even in a dilution of 1/25. Conversely artificial agglutinating sera of Typhoid, Shiga and Flexner were tried against a 24 hour live agar emulsion of bacillus No. 1. At the end of two hours there was no agglutination, but after five hours all the dysentery sera gave a faint trace of agglutination in a dilution of 1/25 and the typhoid serum gave, in addition, a faint trace in 1/50.

An examination of the sera of the patients for agglutinins was made. The serum from case No. 1 was obtained 12 days after the commencement of the illness. With bacilli Nos. 1 and 2 it gave similar results namely marked agglutination in dilutions of 1/25 and 1/50, a trace in 1/100 and a faint trace in 1/200. With bacillus No. 3 there was only a trace in 1/25 and a faint trace in 1/50. The serum from case No. 2 gave with bacillus No. 2 marked agglutination in dilutions of 1/25 and 1/50, and a trace in 1/100. With bacillus No. 1 there was only a faint trace in 1/25 and not even this was present with bacillus No. 3. The serum from case No. 3 gave no agglutination with any of the bacilli. Agglutination experiments with Shiga and Flexner antigens were negative (lowest dilution used, 1/25).

COMMENTS.

The fact that the organisms were present in considerable numbers in the faeces of the first three cases examined (in the first case almost in pure culture), and that two of the three cases showed definite and specific agglutination, makes it appear very probable that this organism was the cause of this small dysenteric outbreak. In addition the organism was not agglutinated by any normal sera that were tried. The exact identity of the bacillus is impossible to determine with certainty. It might be that the bacillus corresponds with the organisms described under the name of *B. coli anaerogenes*—a rather loose term which might include many different organisms. From reference to the literature it appears to correspond closely with the paradysentery bacilli described by Mita as occurring especially in children but occasionally in adults. Mita describes two types of paradysentery bacilli. He states that their colonies on agar are larger than those of true dysentery bacilli, that they are non-motile, do not liquefy gelatin and do not give rise to indol formation. He states also that the older cultures coagulate milk in ten to fourteen days, that acid is formed slowly both in lactose and saccharose, and that whereas type 1 forms acid in maltose and dextrin after one day's incubation, in type 2 the reaction is delayed from one to two weeks. The organism appears also to resemble closely that described by Sonne and considered to be the most frequent cause of dysentery in Scandinavia. Sonne describes a bacillus with rather large colonies showing uneven irregular edges. He states also that mannite and maltose are acidified immediately, saccharose after some days, and lactose later still. There was no formation of indol. Under the title of "Another Member of the Dysentery Group" an organism was described by Duval, and it appears to show some resemblance. This bacillus, although it produced acid in lactose, failed to clot milk. The serum of the patient from which it was obtained agglutinated the organism to a titre of 1/400, the Flexner-Harris bacillus to 1/200 and *B. typhosus* to 1/80. The blood of patients suffering from typhoid agglutinated the organism in dilutions of 1/80 and 1/200. Rabbits inoculated with typhoid bacilli and with the organism produced common agglutinins for both cultures. In the case of the bacillus isolated from this

small outbreak no such affinity to the typhoid and dysentery groups could, however, be demonstrated.

It would appear that, in the investigation of cases of diarrhoea and dysentery, organisms of the slow lactose fermenting type are worthy of attention. Considerable confusion exists in bacteriological literature with regard to organisms of this class. Many bacilli showing cultural characteristics with only slight differences are encountered from time to time in the examination of both faeces and urine. So far those which I have isolated have shown no serological relationship to this organism.

In conclusion I desire to thank Professor L. S. Dudgeon for much help and advice in this investigation.

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ACUTE INFECTION OF THE URINARY TRACT DUE TO A SPECIAL GROUP OF HAEMOLYTIC BACILLI.

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(With 2 Charts.)

IN a paper on *Bacillus coli* infections of the urinary tract, Dudgeon, Wordley and Bawtree (1922)¹ refer to two cases of acute pyelo-cystitis, one of which terminated fatally, due to slow lactose fermenting bacilli which were strongly haemolytic. The diagnosis of paratyphoid fever was considered probable on clinical evidence, chiefly because of the severity of the illness and the prolonged pyrexia which was infinitely longer than occurs in typical cases of acute coli fever. An organism was isolated from each case with identical cultural, serological, and haemolytic properties. On cultural evidence both strains were distinct from true colon bacilli, as they formed blue colonies on litmus-lactose-agar which were similar in appearance to typhoid-paratyphoid colonies, and slowly fermented lactose-broth.

Since this paper was published, I have met with 49 cases of infection of the urinary tract due to this group of bacilli, all of which have run an acute course, in some instances with prolonged fever, and the clinical diagnosis of "enterica" was made in some of the cases. Owing to the appearance of the colonies on litmus-lactose-agar the clinical diagnosis may receive support at the outset, but detailed investigation of the organisms renders the bacteriological diagnosis relatively simple. Some of these cases have occurred in women recently confined, and the same organism has been recovered from the urine and lochia. The urinary symptoms may be masked by the severity of the general reaction which as already stated has led to the clinical diagnosis of "enterica." Two temperature charts are figured to illustrate the types of fever which may occur.

Chart I is from a medical man whose illness ran an acute course for three weeks, while convalescence lasted for about one month. The chief symptoms were pain on passing water, pain in the lumbar region with tenderness on both sides on deep pressure, and severe malaise. There was no doubt that the malaise and general weakness were infinitely greater manifestations of the illness than the urinary symptoms. This patient had suffered from malaria in the Struma Valley during the Great War, but no malarial parasites were

¹ Dudgeon, L. S., Wordley, E., Bawtree, F. (7. XII. 1922). On *Bacillus coli* infection of the urinary tract especially in relation to haemolytic organisms. *Journ. Hygiene*, xxi. 168. (2nd communication.)

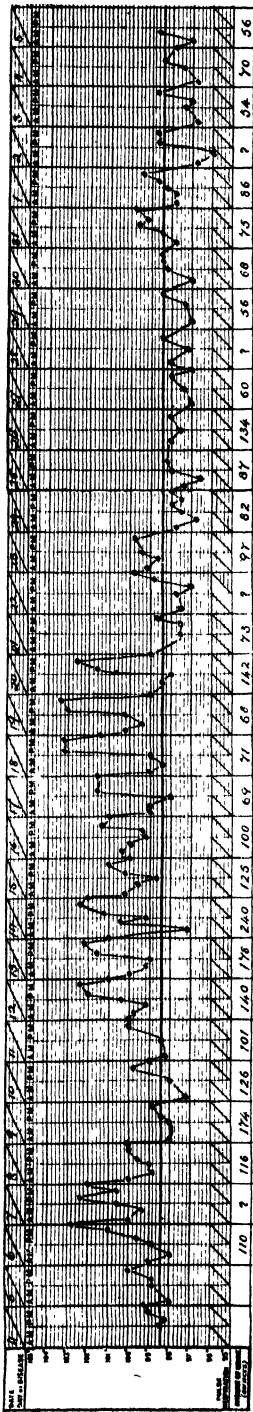


Chart I

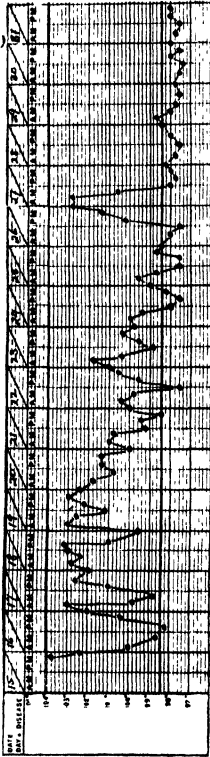


Chart II

found in his blood on this occasion, his spleen was not enlarged, and quinine was ineffective.

The patient's urine contained a thick deposit of pus and bacilli. The serum gave no reaction with the paratyphoid group A, B, and C, and three colon strains. There was a reaction of 1 in 50 to *B. typhosus*, which may have been due to anti-typhoid inoculation, and to his own bacillus. Blood culture was sterile.

Chart II is taken from a woman who developed a sudden acute illness following her confinement. She had twelve days of fever which reached a maximum of 103° F., and was extremely ill during this illness. Here again the general symptoms were so severe as to somewhat mask the urinary infection.

There has been only one fatal case out of the total of 49. The urine from patients who have convalesced from this infection has been found to be sterile, which is in direct contrast to my experience of the usual sequence of events in pure coli cases. Patients infected with this group of organisms, as a rule, are infinitely more sensitive to vaccine therapy than coli cases. Even small doses of vaccine may have the effect of recommencing the pyrexia and the patients' symptoms; so much is this the case that it is necessary to employ considerable discretion in the treatment of these patients with specific vaccines.

Bacteriology. When the urine from these cases was plated on litmus-lactose-agar the colonies for the first 48 hours resembled those of the enterica group, but subsequently with some strains the colonies acquired a greenish-blue colour after a few days' incubation at 37° C. and showed a reddish-tinged centre by transmitted light. Other indicators employed with lactose-agar were neutral-red, phenol-red, and brom-cresol, but no advantage was obtained. In liquid media phenol-red was the only indicator employed.

All the 49 strains have produced marked haemolysis of human red corpuscles in both the stages which have been referred to elsewhere. They were gram-negative bacilli, motile, formed acid and gas in mannitol, dextrose, maltose and dulcitol, although dulcitol was subsequently rendered alkaline by some of the strains. Milk was acidified, and clotting occurred with most of the strains in 72 hours, while a thick or solid clot was formed at the end of one week at 37° C. but with three strains milk became definitely alkaline and no clotting occurred.

Lactose-lemco-broth was acidified during the first 24 hours by some of these cultures, and then became alkaline. Some did not form gas, while some fermented lactose to a slight degree in the course of 72 hours. The majority of the strains did not alter litmus-lactose-agar stab-cultures apart from changing the litmus-blue to a yellow colour. Organism 5659, however, fermented lactose in both liquid and solid media, and 6752 formed typical blue surface colonies on litmus-lactose-agar which finally had a greenish tinge, but it fermented lactose-broth in 48 hours and lactose in agar stab-cultures, and clotted milk. It was the only strain which showed these atypical characters.

Cane-sugar was rendered alkaline with every strain in 48 hours, and the

alkalinity gradually increased up to a period of ten days. A good growth was obtained on gelatine, but this medium was not liquefied.

Table I, showing the cultural reactions of those strains used for the immunisation of rabbits and for the serological tests.

Name or No.	Milk			Saccharose		Jelly	Lactose			Litmus-lactose-agar
No. of days' incubation at 37° C.	1	10		1	10	10	1	6	10	
6489	1	Trace	+2	-	-1	0	-	+	-2	D
Sapsford	2	Trace	+2	-	-2	0	+	+1	-3	D
Duncan	3	Trace	-	-	-1	0	-	+	-	D
6614	4	Trace	-	-	-1	0		+3	+3	D
5659	5	Trace	+2	-	-1	0	0	+2	-1	+1
Winson	6	Trace	+2	-	-1	0	0	+2	-1	D
Williams	7	Trace	+2	-	-1	0	+	+1	-2	D
Ash	8	Trace	+2	-	-3	0	+	+1	-1	D
Northam	9	Trace	+2	-	-3	0	0	-1	+1	D
6752	10	+	+2	-	-2	0	0	+1	-3	+2

+ = acid.

+1 +2 +3 = acid and gas formation in varying gradations, and clotting of milk.

- = alkaline: -1, -2, -3 signifies gradations of alkalinity.

D = Decolorised.

0 = No change.

Blood cultures made into various media have been negative, even when taken at the height of the fever.

Faeces. So far I have not been able to cultivate an organism of this group from the faeces in the acute cases referred to in this paper, but very infrequently such bacilli have occurred in the faeces under other conditions.

SEROLOGICAL REACTIONS.

Patients' sera. The sera from many of the cases were tested with the haemolytic and non-haemolytic coli antigens, with one, two or three of the haemolytic slow lactose strains and with the enterica antigens. No reaction occurred with the coli, typhoid, and paratyphoid A, B, and C antigens, except in the case of those individuals who had been inoculated during the war. No reaction occurred with antigens made from members of this haemolytic group, except in three cases, but here the reaction did not exceed 1 in 400. The absence of reaction in so many cases justified the conclusion that agglutination reactions with human sera and these antigens are of little value in most instances for diagnostic purposes, but a reaction of 1 in 100, or over, is very suggestive of this infection.

The immune sera. The serum from rabbits which had been immunised with vaccines and living cultures of Nos. 5659, 6489 and "Williams," were used for all the serological tests. The rabbits were immunised with formalised vaccines, followed by intravenous injections of the living organisms, or with the living bacilli only. High titre anti-sera were obtained most readily by the intravenous inoculation of rabbits with living organisms. The injections of

live bacilli were commenced with doses of 50 million and then 100, 250 and 500 million, at intervals of one week. No ill effects occurred.

Veal-broth antigens. These were prepared in the usual way by daily sub-culture, killed with 0.1 per cent. formalin, and diluted to 1000 million per c.c. These antigens, however, were seldom satisfactory as, although the end points might remain constant, in many instances the agglutinability diminished rapidly to a considerable degree. The experience gained with these antigens is in direct contrast to that obtained with the true coli antigens which on the whole are perfectly satisfactory.

Agar antigens. These were prepared on the same lines as the veal broth, but were equally unsatisfactory.

Living agar antigens. Living agar cultures grown for 24 hours at 37° C. were found to be the most satisfactory antigens. The agar slopes were inoculated freely so as to cover the entire surface. 2 c.c. of saline were added to each tube ($6 \times \frac{5}{8}$), and the emulsion obtained was filtered when necessary through filter paper. One drop of the emulsion was added to each tube of 1 c.c. of diluted serum, and the whole incubated at 52° C. for 5 hours, and the results read after standing at room temperature for 15 minutes. The method adopted is very similar to that employed in the Weil-Felix reaction. From each strain a veal-broth antigen was prepared, but for reasons already stated it was discarded, and the living agar antigens were used with satisfactory results. Every one of the 49 strains has been found to agglutinate with the three anti-sera employed, and also with high dilutions of these three anti-sera, "Williams," 5659, 6489.

Every strain was tested with a haemolytic colon anti-serum (Dow), but only a very slight reaction was obtained in a few instances. No reaction occurred with T.A.B.C. anti-sera. A non-haemolytic colon anti-serum (5651) did, however, agglutinate some of these strains.

Saturation experiments. An anti-serum, 6489, prepared from one of the cases referred to in Table I, was saturated with one of the cultures "Duncan" referred to in the same table, and as a similar method was adopted in each saturation experiment one description of the details employed will suffice. 2 c.c. of a thick saline suspension of bacillus "Duncan," grown on agar plates, was mixed with 1 c.c. of a 1 in 5 dilution of the immune serum, 6489, and the mixture was left in the ice safe for one week. Each emulsion contained 0.25 per cent. phenol. The mixture was then centrifugalised at high speed, and the control serum and the treated serum compared. The results are expressed as a fraction, the numerator is the result of the treated serum and the denominator the control serum.

The result of the agglutination and saturation experiments point to these organisms as one group of bacilli. Anti-sera prepared from three members of the group agglutinate all strains up to date and de-saturation of these anti-sera has been effected. Anti-coli sera (except no. 5651) do not react with these bacilli except in very low dilutions.

I. I.S. 6489 saturated with bacillus "Duncan." Emulsion 70,000 million per c.c.

	0 ¹
1. On 6489	<u>15,000</u>
	400
2. On Duncan	<u>15,000</u>
	0
3. On Williams	<u>400</u>

II. I.S. 6489 saturated with bacillus "Williams." Emulsion 90,000 million per c.c.

1. On Duncan	<u>2,000</u>
	<u>15,000</u>
	0
2. On 6614	<u>1,000</u>
	400
3. On 5659	<u>5,000</u>
	400
4. On 6489	<u>15,000</u>
	0
5. On Salthouse	<u>400</u>
	0
6. On Sapsford	<u>400</u>

III I.S. 6489 saturated with bacillus "Sapsford." Emulsion 70,000 million per c.c.

	0
1. On 6489	<u>15,000</u>
	0
2. On Williams	<u>400</u>
	0
3. On Sapsford	<u>400</u>

IV. I.S. "Williams" saturated with bacillus "Sapsford." Emulsion 70,000 million per c.c.

	1,000
1. On Williams	<u>10,000</u>
	5,000
2. On 6489	<u>15,000</u>
	2,000
3. On Sapsford	<u>10,000</u>
	400
4. On Duncan	<u>10,000</u>
	1,000
5. On Salthouse	<u>15,000</u>
	1,000
6. On 5659	<u>10,000</u>
	5,000
7. On 6614	<u>15,000</u>

IV a. I.S. "Williams" saturated with bacillus "Duncan." Emulsion 70,000 million per c.c.

	2,000
1. On Williams	<u>10,000</u>
	2,000
2. On 6489	<u>15,000</u>
	2,000
3. On Sapsford	<u>10,000</u>
	2,000
4. On Salthouse	<u>15,000</u>
	1,000
5. On 5659	<u>10,000</u>
	400
6. On 6614	<u>15,000</u>
	0
7. On Duncan	<u>10,000</u>

V. I.S. "Williams" (2) saturated with bacillus 6752. Emulsion 175,000 million per c.c.

	0
1. On Williams	<u>5,000</u>
	0
2. On 5659	<u>400</u>
	0
3. On Sapsford	<u>5,000</u>
	400
4. On 6489	<u>10,000</u>
	0
5. On Winson	<u>2,000</u>
	400
6. On 6752	<u>8,000</u>
	0
7. On 6614	<u>800</u>

VI. I.S. 5659 (2) saturated with bacillus 6752. Emulsion 175,000 million per c.c.

	0
1. On Williams	<u>2,000</u>
	400
2. On 5659	<u>2,000</u>
	800
3. On 6489	<u>2,000</u>
	0
4. On Ash	<u>400</u>
	0
5. On Northam	<u>2,000</u>
	0
6. On Salthouse	<u>400</u>
	0
7. On Struchett	<u>400</u>
	400
8. On Duncan	<u>400</u>
	1,500

VII. I.S. *B. coli* (Dow, Haemolytic Type). Saturated with bacillus 6752. Emulsion 175,000 million per c.c.

On Dow antigen	<u>1,800</u>
	1,800

¹ All these results are expressed as end point reaction which is the limit of agglutination as ascertained with a hand lens. The lowest limit employed was 1 in 400

CONCLUSIONS.

1. A severe acute infection of the genito-urinary tract can be caused by slow lactose fermenting haemolytic bacilli.
2. This infection, as contrasted with acute coli infections, is much less common, more severe, and persists for a longer period, but ultimate complete recovery and freedom from infection is much more probable. Patients suffering from this infection are very sensitive to specific vaccine therapy.
3. No case of chronic infection has occurred up to the present period.
4. Forty-nine cases have been investigated. In every instance the bacilli have been found to be actively haemolytic, serologically similar, and in plate cultures form blue colonies on the surface of litmus-lactose-agar, with delayed fermentation of lactose-broth and alkaline production in saccharose.
5. The organism has not been cultivated from the blood stream or faeces in these acute cases.

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THE CAPILLARY ENDOTHELIUM IN RELATION TO ANTIBODIES.

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(*From the Pathological Laboratory of the Ministry of Health.*)

CONTENTS.

	PAGE
INTRODUCTION	
Importance of endothelium	355
Method of enquiry	359
EVIDENCE FROM EXPERIMENTAL ANAPHYLAXIS	
Laboratory data	359
Comment	364
THE FORMATION OF ANTITOXIN	
Diphtheria antitoxin	367
Comment	369
LOCAL SUSCEPTIBILITY AND LOCAL IMMUNITY	371
DEVELOPMENT OF HYPOTHESIS	
Production of antibodies	374
The nature of antibodies	377
Specificity of antibodies	378
The activities of antibodies	381
DOUBTS AND CONCLUSIONS	
Doubts	384
Conclusions	386

INTRODUCTION.

Importance of Endothelium.

PATHOLOGISTS and physiologists are agreed that the capillary endothelium is of high importance in the animal economy, though it is admitted that its functions are involved in much obscurity. With the immunologist, the primary difficulty is lack of physiological data which would enable him to start with the normal functions and to interpret abnormalities in the light of these. And it is obviously far from easy to plan experiments providing the sort of information the immunologist wants about the functions of endothelium as part of a living mechanism, in which parenchymatous cells, body fluids, and endothelial channels jointly participate.

It would, however, be an exaggeration to say that the functions of the capillary filter in relation to immunity are completely unknown.

In the first place, histologists have devoted much attention to the characters of endothelium in inflammation and in chronic bacterial infections. When histological preparations show visible changes in the morphology and permeability of an endothelial lining, it is safe to assume that these cells are also being

356 *The Capillary Endothelium in Relation to Antibodies*

subjected to finer and invisible modifications, of a chemical and physical nature, which alter their relations to the material which comes into contact with them and to the fluids passing through them.

For example, an endothelial lining forms a barrier which bacteria must penetrate before they can make their way either from the tissues into the circulation or in the reverse direction. Infection and resistance often depend on the impairment or integrity of this barrier. At the focus of infection in a susceptible animal there is usually an inflammatory reaction, which is accompanied by visible alteration in the endothelium and is due to toxic products of the bacteria and perhaps also to the irritant action of disintegrated tissue. A further influence may be operative if the local lesion is bathed in a serous exudate. It is known that such exudates may be rendered toxic by modifications attributable to the action of adsorptive agents. Hence the bacteria, if present in sufficient numbers to adsorb effectively, may transform the exudate into a toxic principle which irritates the endothelial barrier, makes it permeable for the bacteria, and so allows them to gain access to the circulation. This action by adsorption may sometimes help to explain the acquirement of invasive and parasitic powers by bacteria which more usually live as saprophytes on the surface of mucous membranes.

On the other hand, inflammatory change in an endothelial barrier may affect its permeability in such a way that antibacterial fluids, which would be held back by normal endothelium, are allowed to pass into the infected focus and thus assist recovery. This may also help to explain "non-specific immunity"; *i.e.* a foreign protein which is quite unrelated to the protein of the invading bacteria may, by irritating the endothelium, promote the passage of antibacterial substances, which appear promptly, before ordinary antibodies have had time to develop.

Another example may be taken from tuberculosis. Histologically, the relations of endothelium to tubercle bacilli have been the subject of many observations, including those of the Royal Commission on Tuberculosis¹ and of Foot² who traced these cells by the ingenious method of injecting intravenously colloidal suspensions of carbon. Apart from association of endothelium with the proliferative and degenerative tissue changes in tubercle formation, the histologist finds conspicuous differences in the reaction of mammalian and avian endothelium to mammalian and avian tubercle bacilli, and, amongst different species of mammals, differences in the behaviour of endothelium towards the same type of mammalian tubercle bacilli. All this suggests that differences in susceptibility are associated with certain differences in permeability and resistance on the part of endothelium, which have not yet been explained in terms of chemical and physical properties.

In natural immunity against a particular bacterium, *e.g.* the immunity

¹ *Royal Commission on Tuberculosis (Human and Bovine)*. Vol. v. of Appendix to Final Report, pp. 276-83 and 298-300. Cd. 5975. 1911.

² *Journ. Exper. Med.* xxxii. p. 513 and p. 533; xxxiii. p. 271. 1920 and 1921.

of the fowl towards the anthrax bacillus, it is known that antibacterial substances of some sort or other must be present in the body fluids; but they cannot be identified with ordinary antibodies and are not demonstrable in serum obtained by bleeding the animal. The probability is that these substances are extremely labile and are constantly being produced, inactivated or broken up, and formed again in the course of the animal's ordinary metabolism. How is the nature of these substances affected by passage through the endothelial filter of the animal's capillaries? In other words, is the difference between a naturally immune animal, such as the fowl, and a susceptible animal, such as the rabbit, due entirely to primary differences in the body fluids, the endothelial filters being merely passive, or is it partly due to the special selective activity of the endothelium? No categorical answer can be given, because the endothelium and the body fluids are parts of one and the same mechanism, and it is impossible to decide by experiment what functions are attributable to the former alone; but it seems likely, from analogy with filtration by other types of living cells, that the properties of the endothelial filter may differ in the two animals. One may note here the marked difference between the living plasma and the serum, which is removed from the influence of the capillary filter. The fowl's serum is not bactericidal towards the anthrax bacillus, but the rabbit's is, though the former is the immune and the latter the susceptible species.

In addition to the general question of the selective permeability of endothelium in the normal, the infected and the immunised animal of a particular species, there is evidence, from the phenomena of local immunity and susceptibility, that there are local differences in the endothelium of the same animal.

There is another line of investigation which is of much interest. Recent work on anaphylaxis has raised a strong presumption that the endothelium is the site of that union between anaphylactic antibody and antigen which produces shock. Hence one is led to consider whether these cells may not also be concerned with other immunological processes which are not necessarily attended by shock.

The above examples will suffice to indicate that the relations of capillary endothelium to immunity constitute a problem which has definitely come within the sphere of practical interests. One cannot afford to dismiss it on the ground that it is one of the many mysteries of the animal body which appear, at present, to be insoluble, and that, therefore, it would be economy of effort to divert attention to more easily explored channels.

Antigen and antibody are generally used as complementary terms, implying that it is impossible to study them separately; but it is possible to focus more attention on the one than on the other, and I think it is characteristic of most recent work on theories of immunity that interest is focussed on the antigen, the antibody being utilised mainly as a mirror to reflect antigenic properties. I refer to such questions as the serological subdivision of bacterial species.

358 *The Capillary Endothelium in Relation to Antibodies*

What is aimed at is a classification of antigens; the antibodies are, to a large extent, subsidiary to this purpose, in relation to which questions do not arise as to what particular cells (endothelial or other) participate in antibody formation; and, therapeutically, the problem is simply to find the right antibody for the right antigen. Closely associated with this work is the attempt to correlate antigenic structure of bacteria with their virulence. The satisfactory feature of work on these lines is that it is based on laboratory experiments which can generally be planned so as to give unequivocal conclusions, irrespective of problems about the mechanism of antibody formation.

Important as this work is, it needs to be supplemented by the adoption of a different outlook, in which the antibody is of main interest and the antigen is merely a means of stimulating the production of the former or of identifying it. To say that the animal body resists bacterial invasion by the formation of antibodies is a postulate which needs explanation. It is often impossible to demonstrate antibodies in a naturally immune animal; in susceptible animals, successful resistance is not always associated with the production of any known antibodies, and, when it is, it is often impossible to prove that these antibodies are the humoral elements in the mechanism of recovery.

How are antibodies formed and what are their attributes? Once formed, their existence, whilst it continues, is independent of the antigen which stimulated their production, and they must have many characters not comprised within the statement that they were produced by, or will react with, a certain antigen.

These considerations lead to others. What is the real basis of the distinction between "specific" and "non-specific" antibodies? Further, is it correct to assume that the humoral element in resistance to bacterial invasion is always an affair of antibodies, in the current immunological sense of the word? There are also difficulties about the well-known antibodies, such as antitoxins, precipitins and bacteriotropins. They are not always formed as a matter of course and in the anticipated quantity, when a suitable animal is treated with the usual dosage of the "right" antigen; and, when their output is charted, the antibody curve presents curious oscillations, for certain of which no satisfactory explanation has yet been found. All these questions imply that there are serious gaps in what is at present known about antibodies, and that an endeavour must be made to fill up some of them.

In attempting to explore this difficult territory one has to deal with physiological factors, the exact nature of which is largely hypothetical, since there are not sufficient data to translate them into the more concrete terms of chemistry and physics. One must start with some hypothesis, ascertain if it is compatible with known facts, and, if it seems to be, consider whether it helps to explain them.

What cells of the body are most likely to be the site of antibody production? This is an old question; many attempts have been made to associate this function with some particular organ or organs, *e.g.* spleen, liver, lymphatic

glands, etc., but it cannot be said that they have succeeded. As it is known that antibodies may be produced locally in many different sites of the body, it does not seem very likely that their manufacture is the function of any central organ. But endothelium is ubiquitous. That is one reason why it has a particular claim for consideration. This claim does not necessarily imply that endothelium may secrete antibodies in just the same way that special organs secrete special enzymes; antibodies may be the product of a complex interaction between cells (perhaps of more than one type) and tissue fluids. All that need be implied in this hypothesis is that endothelium takes an important, not necessarily an exclusive, part in the formation of antibodies.

Method of Enquiry.

In searching for avenues of approach to an obscure subject, it is useful to start with some idea of what is likely to be helpful.

Filtration is a normal function of endothelium; so it seems to me that, if endothelium participates in the production of immune bodies, its mechanism will probably have something to do with filtration, filtration modified by the adsorption of foreign protein. Do immunological data lend any support to this hypothesis? Preliminary consideration leads me to think that they may do, provided that one can formulate a conception of immune bodies which is compatible with this method of their production.

The idea that antibodies are simply fixed and immutable counterparts of antigens is not sufficiently elastic. Some additional hypothesis is required. I think the simplest is the idea that antibodies in the living body of the actively immunised animal are not necessarily identical, as regards activity and combining properties, with the antibodies demonstrable in that animal's serum, and that the differences, when they exist, are attributable to the transition from the unstable and more complex conditions of the living plasma to the relatively inert and stable conditions of the serum.

These two ideas I have utilised as clues in pursuit of the enquiry.

Proceeding to examine certain data derived from observations on anaphylaxis, the production of antitoxins, and the phenomena of local immunity, I think one can find suggestions which justify a further effort to elaborate the view that the capillary endothelium participates in the production of antibodies.

After developing this hypothesis, I call attention to its limitations in relation to any comprehensive theory of immunity.

EVIDENCE FROM EXPERIMENTAL ANAPHYLAXIS.

Laboratory Data.

These experiments may be described in general terms as studies *in vivo* of the precipitin type of antibody reactions. From the very large mass of work on this subject I propose to select certain data which appear relevant to the present discussion on the production and nature of antibodies.

360 *The Capillary Endothelium in Relation to Antibodies*

From this point of view, the typical experiment in passive anaphylaxis consists in the injection of a foreign protein (A) which is "ear-marked"; it contains a precipitin and hence its relation to the animal's tissues can be detected by subsequent introduction of the corresponding precipitinogen (B), when reaction between the two produces shock. From such experiments it can be shown that A has entered into certain relations with the tissues before B is introduced and it is natural to assume that, as foreign protein, it would have entered into these same relations in the absence of this particular "ear-mark." Thus passive anaphylaxis provides useful information, quite irrespective of the events leading to anaphylaxis, as to the fate of foreign protein when introduced into the animal body parenterally, *i.e.* it reveals something about the behaviour of antigens.

Similarly, active anaphylaxis shows, by means of a shock dose of antigen, that in the process of immunisation antibodies are stored in certain situations. This storage precedes the shock and would occur in just the same way if the conditions necessary for anaphylaxis were not fulfilled. Hence the information about antibodies which is thus acquired is of general utility, the anaphylactic experiment being merely the instrument for its discovery.

These are the two main points which I have in mind in the following references to work on anaphylaxis. My material is derived from Doerr's recent survey of the subject¹.

Many of the long and tedious controversies have now been settled. It is no longer necessary to weigh rival theories as to the humoral or cellular location of anaphylactic shock, nor has any interest survived in the explanation furnished by a mysterious substance called "anaphylatoxin." It is admitted that anaphylactic shock is essentially a cellular phenomenon and that the anaphylactic antibody is a precipitin.

Doerr sees reasons for thinking that the particular cells in which the shock antigen-antibody reaction takes place are not the parenchymatous tissue but the capillary endothelium. Before quoting his arguments, it is appropriate to note that the same suggestion was made many years ago (1910) by W. M. Scott. In his studies of anaphylaxis in the rabbit², he examined the various possible ways in which shock might be produced and arrived at the following conclusions: "By a process of exclusion we are forced to postulate an injury to the capillary walls themselves for the production of the anaphylactic effects. Objective evidence of this is not easy to furnish. Histological methods of greater delicacy than have hitherto been applied are required to bring to the eye such changes in the cells concerned—which cannot, after all, considering the rapidity of recovery, be of a gross character. The prevalence of capillary haemorrhages is of course suggestive."

To return to Doerr, he states that, so far as he is aware, there are no

¹ Weichardt's *Ergebnisse der Hygiene, Bakteriologie, Immunitätsforschung und experimentellen Therapie*, vol. v. pp. 71–274. 1922.

² *Journ. of Path. and Bact.* xv. p. 31. 1910.

facts which are incompatible with this idea of the part played by the endothelial cell, but there are many observations which are in accordance with it. For example, he quotes the experiments of Dale who found that the isolated normal uterus could not be passively sensitised by merely placing it in a bath containing antibody but it was necessary to perfuse the vessels of the organ for 5 hours with dilute antiserum. "Further there are the frequently repeated experiments which show that the organ cells of sensitised guinea-pigs are unable to fix the corresponding antigen *in vitro*. Again, there are the changes of the blood in shock, the predominance of vascular functional disturbances amongst the symptoms of anaphylaxis, etc."

He quotes some interesting observations on the time required for sensitisation in passive anaphylaxis. Guinea-pigs were sensitised intravenously with the minimal dose of antiserum and during the first stages of the latent period about half their total volume of blood was removed and replaced by normal blood. If the withdrawal of blood took place within the first hour, the anaphylactic condition was not produced; removal after the lapse of one hour or at a later period had no effect; the animal became as markedly hypersusceptible as the control which had not been bled. Owing to the shortness of time required for the union of cells with the serum containing anaphylactic antibody, it appears to Doerr improbable that the antiserum passed through the vessels and entered the parenchymatous tissue; "one would rather think of the capillary endothelium as the site of union, of activation, and of the antigen-antibody reaction."

It has been estimated that the amount of antibody taken up by the cells during the first hour is about 40 per cent. of the quantity injected; the surplus remaining in the blood apparently plays no further part in the sensitising process. After this first union, there is the well-known latent period (24 hours or less) before the anaphylactic condition is established. During this time "activation" of the cellular antibody is brought about. Doerr remarks that this second phase does not mean that time is required for the gradual accumulation of antibody up to the requisite concentration, since Fenyvessy and Freund had shown that the latent period was not abbreviated if 3-4 multiples of the minimal dose of antiserum were injected; with the larger dose, it was proved by titration that about 40 per cent. of the total was fixed by the tissues in one hour, *i.e.* a much larger quantity than after the minimal dose. According to Doerr, the "activation," for which a latent period is required, does not imply that a qualitative change has been produced in the antibody but that there has been a slow transition (as is commonly the case in colloidal reactions) from loose to firm union between antibody and the shock cells.

The sensitising antiserum is a foreign protein and its antibody component does not split off from the rest of the protein when the serum is taken up by the cells; both disappear from the circulation simultaneously. But Doerr does not think it probable that the foreign protein passes through the cell membrane and permeates the cell protoplasm, because, if it did, it would

362 *The Capillary Endothelium in Relation to Antibodies*

damage the cell and be incompatible with the latter's continued vitality; but passive sensitisation does not lead to extensive tissue necrosis; in fact hypersensitiveness depends on the maintenance of the vitality and reactive capacities of the sensitised cells. Hence he concludes that what actually takes place is attachment of the antiserum to the surface of the cell by adsorption, "activation" being a secondary fixing of this adsorbed material.

In spite of careful experiments by Doerr and others, it had been found impossible to obtain "reversed anaphylaxis," *i.e.* the passive anaphylactic shock cannot be produced if the antigen is injected first and the antibody afterwards. What is the reason? Is the antigen modified by the cells in such a way that it becomes incapable of subsequent reaction with antibody? Or is it because the antibody has to participate in some change before its union with antigen can provide the stimulus causing anaphylactic shock? Doerr adopts the latter view, the postulated change being the "activation" referred to above. "Only the antibody enters into that intimate relation to the shock cells which is necessary for the reaction, not the antigen."

Experiments on "antisensitising" are of interest. It has been shown that guinea-pigs can no longer be passively sensitised with rabbit immune serum if they have previously received subcutaneous injections of normal rabbit, sheep, dog or human serum. After small doses of serum, the protective action is produced slowly (after 8 days); large doses (8 c.c. spread over 4 days) produce resistance more quickly (in 1-3 days after the last injection). When the refractory condition is once established, it persists for a considerable time (at least for 68 days). What is the explanation of this phenomenon? As the reaction is non-specific, one can hardly postulate the formation of "anti-antibodies." Doerr is inclined to the view that the guinea-pig's cells were "saturated" with the first doses of foreign protein, so that the subsequent immune serum could no longer enter into relation with them. In support of this view he quotes experiments of Weil's, who showed that, though guinea-pigs treated with large doses of rabbit, sheep, or human serum resisted heterologous passive sensitisation, they could be passively sensitised without any difficulty when homologous antiserum was used (the serum of hypersensitive guinea-pigs). Apparently the animal's cells had no difficulty in taking up the antibody contained in the proteins of their own species.

Doerr discusses the view that, on the cellular theory of anaphylactic shock, circulating antibody must be protective, by neutralising antigen on its introduction and before it can unite with antibody localised in the cells. In support of this, Weil showed that actively or passively immunised guinea-pigs could be made insusceptible to the introduction of antigen if a good dose of rabbit antiserum were introduced intravenously shortly before the antigen. These results were confirmed by others, but it was found that their significance was open to question. Friedberger and Hjelt showed that, if guinea-pigs were sensitised with anti-horse or anti-cat serum obtained from rabbits, the anaphylactic condition could be temporarily put in abeyance by inoculating

1 c.c. of normal rabbit serum 24 hours after the passive sensitisation. Doerr quotes a similar instance from active anaphylaxis, where this effect was produced by rabbit serum but not by the serum of other species. The effect persisted for at least 15 hours in active anaphylaxis and for at least 24 hours in passive anaphylaxis; it was demonstrable very soon after the introduction of the antagonistic normal rabbit serum. In view of such non-specific effects, one has to be cautious about postulating a protective action of circulating antibody. "In the much greater proportion of cases, free and cellular antibody co-exist in the organism, both in active and in passive anaphylaxis; only the relative quantities of the two vary within wide limits." Moreover, "the neutralisation of precipitinogen by antibody is not completed either suddenly or without leaving a trace behind. Even if antibody is present in considerable excess, free antigen remains in the mixture and may enter into reaction with freshly added antibody or with antibody which is differently located."

The condition of (1) passive susceptibility to anaphylaxis, when conferred by injection of heterologous antiserum, persists unaltered for some days; the hypersusceptibility then diminishes and finally disappears from the 6th to the 10th day. But, if (2) the antiserum is homologous, the condition lasts from 60 to 70 days. In (3) active anaphylaxis it generally remains for more than a year. (1) may be explained by the assumption that the foreign protein, together with its contained antibody, becomes inactive as soon as the organism has formed an antibody against this protein. Hence "a co-existence of antigen and antibody is possible *in vitro* and in the blood-stream but not in the cells, where one of the reacting components excludes the presence of the other." In (2) the protein is homologous, so there is no reason why it should be turned out of the cell so readily. But why the marked difference between (2) and (3)? Doerr admits that this is a puzzle and discusses it as follows: "Is there such a great difference between the protein of species and of individual? Or is the mode of union between cell and antibody introduced from without less firm than between cell and autochthonous antibody? And what is meant by autochthonous? Does it mean that the antibody only arises in the like organism or that it arises in the anaphylactically reacting cells? Site of production and localisation of antibody are not necessarily identical; even in actively sensitised animals, the antibody might, for example, be produced in the lymph nodes and then form a secondary attachment with the smooth muscle fibres, so that the reacting tissue would be only passively sensitised."

In concluding these quotations from Doerr's article, I note that he makes a suggestion which I have already raised at the commencement of this section. Passive anaphylaxis might be utilised to study the fate of foreign protein in the animal body. "Its suitability for this purpose is provided by the circumstance that antibody is united with protein and is broken up along with the latter and that the anaphylactic antibody is the only one which admits the ready demonstration of its presence in, or attached to, fixed tissue cells."

Comment.

In dealing with general questions of immunity, it is not quite clear whether anaphylaxis should be treated as a main problem or as a side issue. Until recently, the tendency has been in the former direction, and there has been much theoretical discussion about the relation of anaphylaxis to "Allergie," which, being an altered state of tissue reaction caused by the introduction of foreign protein, was supposed to be the preliminary stage of immunisation. Since then a reaction has set in. Theories about "Allergie" have tended to assume a merely academic interest. It is realised that a good deal of the controversial work about "anaphylatoxin" and the like has been wastage, and that one may rest content with the view that shock is due to a reaction between precipitin and precipitinogen which takes place in certain cells of the animal body. Hence the tendency to leave anaphylaxis alone, as being an interesting but not specially important phenomenon, the nature of which has been duly explained.

Whichever view be adopted, there is one curious feature which is worth considering. How is it that, unless obvious precautions be neglected, accidental anaphylaxis is a rare event? As Doerr has shown, it cannot be because there is usually a supply of circulating antibody which neutralises the antigen before it can reach the cells. Shock may occur in spite of the presence of such circulating antibody; on the other hand, shock may be absent under conditions where there is antibody in the tissues but not in the blood stream. Amongst the many factors upon which absence of anaphylaxis in active immunisation probably depends, is one of them attributable to a difference between the condition of antibody in the serum and its condition in the plasma and cells of the living body? If there is a difference between the two, the latter condition may not always be appropriate for a precipitinogen-precipitin reaction, and anaphylaxis may only occur when the cellular antibody is of the serological type.

Doerr, if I interpret him rightly, does not see any need for this hypothesis. He states that antigen and antibody may co-exist *in vitro* and in the circulation but not in union with cells. This may be true in some cases but it is rather puzzling in others. Admittedly, the work on anaphylaxis provides strong evidence that a precipitin and its antigen cannot co-exist in union with the same cell as independent and compatible entities, since anaphylactic shock would take place. But how are antigen and antibody kept apart in active immunisation, when shock does not occur? One must suppose that the antigen comes into close contact with the cell which it stimulates to form antibody. How is it possible, during the continuation of this process, to avoid contact between antigen and cellular antibody? It would seem arbitrary to postulate that special colloidal conditions prevent that sort of contact which would lead to interaction, and I think some other kind of explanation is needed. Just as certain physiological products, such as enzymes, may have an antecedent form

which is not active, so it is possible that the antibody, before being turned out of the cell, may be in a quiescent stage which precedes its conversion into a precipitin.

If there are these two phases in the constitution of an antibody, does the change from the first to the second take place in the circulating plasma or in the serum? If the former alternative is correct, there would appear no reason to doubt the ordinary serological tests for circulating antibody. But if the change does not (or may not) take place until the serum has been collected, there will obviously be a different explanation for the apparent co-existence of circulating antigen and antibody in the actively immunized animal; the antibody may not have assumed its precipitin type.

Some information about the behaviour of antigen can be obtained from experiments in passive anaphylaxis where a foreign protein, which must necessarily behave as an antigen, can be identified owing to its content of anaphylactic antibody. It appears, from this method of observation, that with some antigens adsorption by the cells takes place very rapidly (in about one hour), that a change, probably a firmer union with the cells, occurs during the first 12 hours or thereabouts (latent period in passive anaphylaxis), and that the adsorbed antigen is broken up and presumably ceases to function as antigen in from the 6th to the 10th day (termination of anaphylactic condition). As the cells are not damaged by these events, there is a strong probability that the foreign protein does not penetrate them but simply acts on their surface.

Can anaphylaxis provide any further information about the effect of this action upon the cell? The impossibility of obtaining "reversed anaphylaxis" shows that the first foreign protein modifies the cell in such a manner that the latter does not unite with the second protein (containing "anaphylactic antibody") in the same way, since it no longer makes with it that firm union which is the necessary prelude to shock. Again, the "antisensitising" experiments show that in the course of a few days (five or more) the first foreign protein impresses a definite change on the cells which have adsorbed it. They behave in a different, though non-specific, manner when the second protein is introduced, the evidence being, as before, that they do not make with the latter the firm union necessary for shock. The postulate that the cells are "saturated" with the first protein seems unsatisfactory. This explanation is not valid when the carrier of the precipitin is not foreign but homologous protein, since shock is obtainable under these conditions; and it is difficult to imagine how the first foreign protein could remain linked to the cells (still less "saturate" them) for so long a period as 68 days. Moreover, there is no evidence that the cells of an animal which has formed antibody in response to a particular antigen are "saturated," since the animal is quite capable of producing other and different antibodies when their corresponding antigens are subsequently injected.

I think the main conclusion is that cells which have adsorbed foreign protein are changed in some way which has not been clearly defined, and that

366 *The Capillary Endothelium in Relation to Antibodies*

this change tends to persist after the disappearance of the foreign protein. This evidence, derived from anaphylaxis, of the acquirement of a new property by the cells is in accordance with the fact that cells which have ceased to turn out a particular antibody may be caused to renew their supply of it by the injection of a non-specific stimulus.

Why is the condition of anaphylaxis produced by active immunisation of much longer duration than the passive condition induced by injection of homologous antiserum? In the former case, the protein constituents within the cell which are called "anaphylactic antibody" are living material which is renewed from time to time in the course of the cell's metabolism; in the latter case, the protein which acts as antibody is dead and therefore incapable of renewal, though, being homologous and non-irritant, it resists disintegration for a much longer time than foreign protein would. If this is a satisfactory explanation, it may have a more general application to immunity. Is the long period of immunity which follows infection or vaccination with certain viruses due to the creation of living cellular antibodies which propagate themselves? And when susceptibility returns in a relatively short time after recovery, does it mean that the antibodies, though built out of the animal's own protein, are incapable of self-renewal as living material and therefore disappear in the normal processes of protoplasmic repair?

Doerr's reasons for thinking that the endothelial cell is probably the site of anaphylactic shock are of obvious interest in relation to the wider questions of the immunological functions of endothelium, provided that one does not place undue weight upon the data. The suggestions which they furnish do not amount to proof. As he points out, mere evidence that antibodies are closely associated with endothelium does not exclude the possibility that they were manufactured elsewhere and subsequently adsorbed by these cells. All that can be said definitely is that anaphylaxis provides information about the interactions between foreign protein and certain cells of the body and that this information does not appear incompatible with the hypothesis that endothelium participates in the formation of antibodies.

The anaphylactic test, as has been noted, provides an ingenious method of "ear-marking," and thereby identifying, a foreign protein which is used as an antigen. One naturally asks whether it is possible to find other methods of achieving the same object. I wonder if the biochemists will ever be able, by using an artificial antigen containing a known chemical substance with specific antigenic properties, *e.g.* an iodo-protein, to identify that substance by microchemical methods in histological preparations of the immunised animal's endothelium. If so, the microchemical reactions at different stages of immunisation might throw some light on the mechanism of antibody formation.

THE FORMATION OF ANTITOXIN.

Diphtheria Antitoxin.

It is of interest to consider whether the hypothesis now under discussion is compatible with what is known about the formation of antitoxin. I take diphtheria antitoxin, as being the best known and the most important. There is also the advantage that the data which are of special interest in this connection have been recently reviewed by Madsen in his first Harben Lecture for 1922¹ on specific and non-specific formation of antibodies.

Madsen raises several important questions about the reactions of a horse which already possesses some diphtheria antitoxin in its blood.

When a horse receives a further immunising dose of toxin, there is a "strongly pronounced negative phase of three days' duration, after which the antitoxin concentration rises until it reaches a maximum on the ninth day, and then falls again." On the ninth day the formation of antibodies has "practically ceased" and "the factors destructive to the antibodies can now have full play." The destructive factors, he states, have been present all the time but have been concealed by excess of production. "This phenomenon, shown by the active immunity curve, may be compared with what is observed if one throws a stone upwards into the air; the force of gravity, which is continually acting upon it, is counteracted by the upward driving force, but only up to a certain height, when the stone will again fall down."

Why is injection of toxin into such an animal followed by the appearance of the negative phase? It cannot be explained by the hypothesis that part of the antitoxin present is neutralised by the new dose of toxin. "A simple calculation will suffice to show that if we have to deal with a horse possessing, for instance, a concentration of about a few hundred antitoxin units in its blood, a few c.c. of this will suffice to neutralise even the maximum amount of highly potent diphtheria toxin which we are able to inject into the horse." Madsen points out further that the maximum decrease is not observed at once, as would be expected if the antitoxin were neutralised, but not until one or two days after injection. He suggests that the effect may be due to "some inhibitory action on the antitoxin-producing cells."

Again, why is this fall in antitoxin followed by a rise? This fact also is obviously incompatible with the hypothesis of neutralisation such as occurs *in vitro*. "If a few c.c. of blood are withdrawn from the horse and mixed with the toxin, and this again injected into the horse, no reaction occurs, either in respect to fever, local infiltration, etc., or to antitoxin formation." There is a corresponding difference between active and passive immunity. The actively immunised horse reacts strongly to a fresh subcutaneous injection of toxin "both in regard to general reaction and production of antitoxin," whereas the passively immunised animal, possessing only a slight concentration of antitoxin, shows no reaction to the same amount of toxin.

¹ *Journ. State Med.* xxxi. Feb. 1923.

368 *The Capillary Endothelium in Relation to Antibodies*

Madsen considers that the most satisfactory explanation of antitoxin formation "seems still to be the view advanced by Salomonsen and myself in 1896, namely, that *the cells of the organism under the action of the toxin may be supposed to acquire a new functional capacity, that of secreting antitoxin*, each new injection of toxin acting as an incitement to antitoxin production."

He then proceeds to point out that the injection of specific antigen is not the only means of obtaining an increased production of antibody. It may be brought about by a variety of non-specific stimuli, such as removal of existing antitoxin by repeated bleedings, the production of an inflammatory condition, or the action of various chemical substances. "On the basis of the conception of antitoxin formation as a sort of secretory process, Walbum thought it probable that it might be acted upon by substances possessing a catalytic action, such as, for instance, certain *metallic salts*, and by a closer study this proved to be the case." The remainder of Madsen's lecture is mainly occupied with records of experiments which illustrate this point.

In the first experiments on horses the endeavour was made to estimate the influence of one of these salts, manganese chloride "*after the action of the toxin had ceased to prevail*." When the antitoxin content of a horse had fallen to 160 units per c.c., small intravenous doses of the salt were given daily. "The antitoxin concentration showed a rise from 160 a.u. to 350 a.u. per c.c. as a consequence of the metallic salt alone, without toxin." In another example, the antitoxin content was raised from 700 to 1000 units.

Similar effects were observed when horses received continued treatment with both toxin and the metallic salt. A horse had persistently refused to yield a maximum of more than 200 units when treated with toxin alone; when daily injections of manganese chloride were commenced "*simultaneously with immunisation with diphtheria toxin in the usual way*," a content of 375 units was eventually obtained. This case was corroborated by others, which justified Madsen in making the general statement that "there is thus no doubt that by the application of metal salts, more especially manganese chloride, we are able to produce a rise in the antitoxin production."

Another point of great interest is the rapidity with which various metallic salts produce their effect. Referring to an example from his antitoxin curves, he says: "It will be seen that, following a single injection of magnesium chloride the antitoxin content of a horse immunised shows an enormous rise, reaching the maximum in the course of one hour. The rapidity of this rise is quite startling, and the relation is here quite different from that of the usual antitoxin curve appearing after toxin injection, since the negative phase is totally absent." Madsen considers that these experiments support the conception of antitoxin formation as being a secretory process, though he regards it as an open question whether it is possible to form new antibodies with such rapidity or whether the real explanation is that antibodies previously lodged in the organism are suddenly pushed out into the circulation under the action of the salt.

In addition to his observations on antitoxins, I may note that Madsen also calls attention to the stimulating effect of metallic salts on the production of other types of antibodies, *e.g.* agglutinins.

In concluding this outline of Madsen's lecture, I ought to add that he duly recognises the difficulties of his subject. "It cannot be denied that in spite of an enormous amount of work and a vast accumulation of facts, we still remain without the deeper understanding of some of the principal problems regarding immunity. This is, for instance, true of the question of the formation of the antibodies....The *negative phase* appearing in the curve representing active diphtheria antitoxin formation is still a mystery....It is just as difficult to find an explanation of the fact that this decrease in antitoxins is succeeded by a rise." I think these quotations will suffice to show that Madsen is careful to avoid any appearance of dogmatic finality.

Comment.

Madsen's explanation of his data is cogent and undoubtedly coincides with the views of the majority of immunologists. It is largely based on the assumption, which I admit to be the orthodox opinion, that serological tests furnish an accurate record of circulating antibodies. Still, this assumption is not absolutely proved to be correct; in some cases, if not with diphtheria antitoxin, serological reactions *in vitro* do not necessarily run parallel with immunological reactions *in vivo*. Therefore, as there are still some points about this antitoxin which have not been finally settled, it may be permissible to consider a different hypothesis, which is neither proved nor orthodox.

In the tissues and in the circulation there may be an antecedent form of that antitoxin which is demonstrable in the serum. This antecedent form may vary in its stability in different phases of the animal's immunological history, yielding more antitoxin in the serum when more stable and less when less stable.

On this latter view, the negative phase which is observed when a horse, already immunised or partially immunised, receives a fresh dose of toxin might be interpreted as follows. The new toxin is rapidly adsorbed by the cells which play a dominant part in the output of antibody. This union between toxin and the surface of the cell becomes firmer in the course of the first day (as in the case of foreign protein carrying anaphylactic antibody) and then causes a brief disturbance in the cellular mechanism, with consequent increase in the instability of the antibody which passes into the circulation. The result is a decrease in serological antibody but not a quantitative decrease in the less stable circulating antibody.

In the next phase, from about the third to the tenth day, the disturbance has subsided and the effect of cellular union with the new toxin is a quantitative increase of circulating antibody, accompanied by an increase of that proportion of it which will assume a stable condition in the serum.

After about the tenth day, the adsorbed toxin begins to be broken up and

370 *The Capillary Endothelium in Relation to Antibodies*

this change in the cellular mechanism is marked by a diminution in the proportion of antibody which will become stable in the serum, though not necessarily by a quantitative diminution in circulating antibody.

This hypothesis seems to me rather less difficult to understand than the postulate that the same substance (the new toxin) can produce two opposite effects, first inhibition and then stimulation of the secretion of antitoxin.

The condition after the tenth day may be altered by non-specific means, their effect being, according to the above hypothesis, not to increase the total of circulating antibody but to modify its character, so that a greater proportion of it is capable of becoming stabilised in the serum.

This would involve the assumption that the catalytic action of a metallic salt, such as manganese chloride, can increase capacity for stabilisation, an explanation which seems to me less hazardous than the postulate that such substances actually stimulate the production of specific antitoxin *de novo*.

It has been shown by work on anaphylaxis that foreign protein can be adsorbed by the cells in one hour. This also appears to be the case with such substances as manganese or magnesium chloride, in the work recorded by Madsen. Proteins are highly complex and their interactions with the cell form a chain of events for the completion of which many days are required. With simple substances such as metallic salts the conditions are different. Here it is natural that the full catalytic effect of the reagent should be felt as soon as union with the cells is effected, and that it should diminish as soon as the salt begins to be eliminated from them. Hence the difference between toxin and metallic salts in their influence on the antitoxin curve.

Madsen states that the formation of antibodies has "practically ceased" on the ninth day after injection of toxin and that thereupon factors which destroy antibodies "have full play"; manganese chloride, however, will promptly renew the antibody content. It will be seen that the above hypothesis attempts to provide a different explanation. Perhaps further light will be thrown on the subject when these interesting observations on the action of metallic salts have been extended and confirmed.

The admittedly unorthodox view which I have put forward may be considered in relation to some of the puzzling facts concerning the co-existence of antigen and antibody. Why is it that toxin is neutralised by antitoxin *in vitro* and in passive immunity (and thereby loses its antigenic powers) but not in the circulation of an actively immunised animal? My suggestion is that in the last case the circulating antitoxin, unlike the serological antitoxin, is too unstable to form that firm union with toxin which would abolish the latter's antigenic function. It does not follow that the "active" antitoxin is indifferent to the toxin; it may form loose colloidal or chemical union with it, which would suffice to prevent the latter from exerting its full toxicity on the cells, and so would explain why an immunised animal is more tolerant of toxin than a normal animal.

I have a brief comment to offer on Madsen's secretory theory. If one starts

with (1) the view that antibodies are cellular secretions due to the stimulus of the specific antigens, one is inevitably led to consider in what way the antigens produce this effect. In such considerations, it seems to me, one cannot avoid the postulate that (2) the antigen must be brought into intimate contact with the cell, probably by adsorption, and must also modify the fluids which permeate the cell, probably by catalytic action. One might go on to amplify these considerations, but at this point I wish to call a halt. Hypothesis (2) is evidently contained within the wider hypothesis (1).

Why not try to economise hypotheses by starting with the smaller one (2)? It seems to me possible that considerable progress may be made with (2) alone. Perhaps it may not be necessary to encumber oneself with (1), which, apparently, would involve difficulties similar to those of Ehrlich's theory regarding the production of antibodies in excess of the cell's requirements.

Madsen's lecture raises another question about economy in hypotheses. If a stone is thrown into the air, it is controlled by the force of gravity during the whole of its course. But is there an equally valid law of nature which compels the animal body continuously to destroy the protective antibodies which it is laboriously manufacturing? Such rapid and continuous destruction would be something quite different from the normal using up of material (followed by its replacement) which is associated with living matter; and I should hesitate to agree that Madsen's postulate is a general law governing immunological processes.

Perhaps some simpler explanation can be found for the oscillations in the serological antibody curve during immunisation. It is known that the transition from life to death is soon followed by a very profound change in the body fluids. These serological antibodies are dead material; the living matter from which they were derived has undergone change, and many of its attributes have been destroyed. Instead of assigning the act of destruction to a constant and normal function of the living body, would it not be more plausible to explain it as being largely, though not entirely, due to the changes accompanying the transition from living to inert matter? This would lead to the less ambitious hypothesis, which I have suggested above, that the living material from which the serological antibodies are derived varies qualitatively (as regards capacity for becoming stabilised) as well as in quantity of antibody content, and that oscillations in the serological curve are partly attributable to the former type of variation.

LOCAL SUSCEPTIBILITY AND LOCAL IMMUNITY.

The main facts are the ordinary observations of clinical medicine. In most bacterial infections the clinical picture is an example of local susceptibility and local immunity; infections are distinguished from each other by the differences in the details of the picture, the distribution of the lesions, their characters, and their capacities for recovery.

372 *The Capillary Endothelium in Relation to Antibodies*

The explanation of these differences is the task of pathology, and one part of the problem is to consider the functions of endothelium. How does this tissue, which is ubiquitous, participate in these manifestations of local susceptibility and local resistance? This question again must be subdivided into several, the one of immediate concern here being the question of endothelial participation in the local formation of antibodies.

As to the nature of such possible participation, it must be remembered that, though the endothelium of the blood and lymph channels may be regarded collectively as an important organ regulating the functions of the body, it is not an independent organ; its normal activities depend on the special characters of the adjacent tissues, and its capacities for dealing with bacteria also depend on local conditions. In any given locality, the endothelium, the fluids on either side of the endothelial barrier, and the tissues which depend on these fluids for their vitality act in conjunction as one mechanism. Thus there are local differences in endothelium, but, as these depend on environment, it does not seem likely that the characteristics of endothelium alone will fully account for the formation of antibodies; and for a similar reason it does not seem possible to regard cells of epithelial or other type as independent agents which are entirely responsible for the production of antibodies.

Some light on the clinical data, though perhaps not very much, is thrown by laboratory experiments.

It is customary in the text-books to quote a famous experiment by Wassermann and Citron on a rabbit. They injected typhoid bacilli subcutaneously into its ear, immediately applied a ligature to the base of the ear, and kept the ligature in position for several hours. After nine days they determined the bactericidal titre of the animal's serum and then amputated its ear. After this operation they found an immediate and rapid fall of antibody and inferred that the chief source of antibody formation had been removed. Many other experiments have been recorded which suggest that, in some circumstances, antibody is produced locally at the site where the antigen was injected. And this view is in conformity with the fact that failure has attended the numerous attempts to show that the general function of turning out antibodies is allocated to some central organ or tissue.

There is also laboratory evidence that the conception of local immunity must not be narrowed down to the idea that for the production of this condition some one type of cell, differing with different bacterial infections, is entirely responsible.

For example, if Shiga bacilli are introduced into the alimentary canal of a suitable animal, the intestinal mucosa is the susceptible tissue which is attacked. If, instead, the bacilli are injected into the peripheral circulation of a similar animal, the intestinal mucosa is again the site which is selected for attack; but in this case the bacilli must penetrate the capillary endothelium adjacent to the mucosa before they can reach the latter, and it is this endothelium which is, primarily, the susceptible tissue. If the above two experiments

are repeated on an animal which has been immunised by parenteral inoculation, both epithelium and endothelium of the intestinal area are found to be resistant. In all these events, as it seems to me, susceptibility or resistance must be attributed to the mechanism as a whole, not simply to a quality of one of its factors, epithelium, endothelium, or body fluids. And, though the susceptibility or immunity is local in the sense that it is manifested in the intestinal wall, it is not altogether local, since one of the factors in the mechanism consists of material derived from the general circulation.

Some further points of interest are raised by F. P. Gay. In a recent article on local and general immunity¹, he endeavours to distinguish "true local immunity" from mere evidence of the localisation in certain areas of antibodies derived from the general circulation, *i.e.* merely local mobilisation of a general form of protection. He defines "true local immunity" as an "Umstimmung" or "retuning" of the tissues, which causes them to react in a new fashion, and quotes some experiments with streptococci as affording evidence of this condition. A strain of fixed virulence was used and it was found that it regularly produced erysipelas in rabbits when inoculated intradermally in a dose of 0.1 c.c. The animals made complete recovery, although double the dose would produce fatal septicaemia. After recovery, the animals were completely immune against re-inoculation intradermally elsewhere on the body. But they were not protected against intravenous inoculation with the same dose, although "the minimal lethal dose is practically the same intravenously as is the symptomatic dose intradermally." Conversely, he found that "intravenous inoculation of sublethal doses protects the animal against intravenous inoculation but not against intradermal inoculation." Differences corresponding to these were found in the effects of intrapleural as compared with intravenous immunisation.

As regards dosage, these experiments illustrate a difference between unsuccessful and successful resistance which may perhaps be explained in this way. When the first intradermal or intravenous dose exceeds 0.1 c.c. the irritant action of the cocci ultimately breaks down the endothelial barrier, the result being generalised and fatal dissemination. With the correct immunising dose, this does not occur; the bacterial antigens are adsorbed on one or other side of the endothelium and cause this barrier to resist penetration by a larger dose which would be fatal to a normal animal.

One feature of the endothelial filter is that chemical and physical conditions on the one side of it (the lumen of the vessel) are not the same as those on the other side, which is in proximity to the tissues. Is this exemplified in the different effects of intravenous and intradermal immunisation? Since cutaneous immunisation at one site protects the whole of the animal's skin, the protective substances must get into the circulation, in order that they may be distributed throughout the skin area. Therefore it seems necessary to postulate two different kinds of protective substances, the one produced by cutaneous and the other

¹ *Journ. Immunol.* VII. p. 1. 1923.

374 *The Capillary Endothelium in Relation to Antibodies*

by intravenous injection. These differences are possibly attributable to differences in the character of the one or other side of the endothelial barrier which has absorbed the antigenic material. There is, however, an alternative supposition. In order to act as antigens, bacteria must be broken up, and the exact way in which they are broken up may depend on their particular location, *e.g.* in the skin or in the circulation; so it may happen that the antigens produced in the former situation differ, in some respects, from those produced in the latter, and that these differences are reflected in their antibodies.

Partial streptococcal immunisation may, in one of its aspects—immunisation against septicaemia without protection against erysipelas—bear a resemblance to some results obtained by Cecil and Blake in experiments on monkeys with killed pneumococcus vaccine. They found¹ that subcutaneous vaccination gave definite protection against experimental pneumococcus septicaemia, but did not protect against pneumonia produced by intratracheal infection. This limited degree of immunity they described as “humoral.” The vaccine given subcutaneously evidently found its way into the blood stream; it may have modified the side of the endothelium facing the circulation, caused it to produce antibodies with particular characters, and increased its resistance to penetration by circulating pneumococci. In intratracheal infection, the lung would have to deal with pneumococci on the tissue side (which had not been immunised) of the endothelium.

The above examples prove nothing; and I do not think it would be possible, by adding to their number, to provide adequate data for the induction of any general principle. All that may be claimed for them is that they indicate the presence of many factors in local reactions to bacterial invasion and raise questions about endothelium without providing any definite answer². After all, the clinical rather than the experimental data are the main facts requiring explanation.

DEVELOPMENT OF HYPOTHESIS.

In the preceding sections I have endeavoured to prepare the way for the following elaboration of ideas about the capillary endothelium in relation to antibodies. If any hypothesis of this nature is worth considering, one wants to know more definitely what shape it is going to take.

Production of Antibodies.

The ordinary and admittedly vague idea of an antibody is that it is something (*e.g.* a precipitin or an agglutinin) which is produced in the animal body by the action of foreign protein (antigen) and reacts with that protein both *in vivo* and *in vitro*.

The current explanation of the way in which antibodies are formed is equally vague and tentative. It is commonly supposed that the stimulus of

¹ *Journ. Exper. Med.* xxxi. p. 519. 1920.

² For Bearedeka's views on local immunisation, his articles in the *Bull. de l'Inst. Pasteur* (xx. p. 473 and p. 513, 1922) may be consulted.

foreign protein causes certain of the animal's cells to protect themselves against injury by secreting something (the antibody) which acts as an enzyme towards this irritant foreign protein and digests it. There is no need to quarrel with this idea if it is taken merely as a suggestion and not as a literal statement of fact. There is certainly some resemblance between the stimulus of a foreign protein, introduced parenterally, and the stimulus which causes the secretion of enzymes by the digestive tract; and the action of an antibody on its antigen may, to some extent, resemble the catalytic action of a ferment. But it is very far from being proved that antibodies are ferments in the strict physiological sense of the term, or that they are primarily and essentially a cellular secretion comparable to the characteristic secretions of special glandular structures.

Hence it is not necessary to postulate that antibodies are the product of cells naturally endowed with a special secretory capacity; if it were, it would certainly be difficult to imagine why endothelium should possess this characteristic in preference to any other type of cell.

If, however, one thinks of antibody formation not as a special secretion but as a process which has something to do with filtration and results in a modification of the filtered fluid, then endothelium has a particular claim for consideration, because filtration is a specially important function of this type of cell.

Some idea of the delicate selective activity of a cellular filter may be derived from the work of Hamburger and his associates on the permeability of the glomerular epithelium of the frog's kidney¹. They showed, by perfusion experiments with various sugars, that permeability or retention did not depend upon the size of the molecules but upon their precise stereo-chemical configuration. For example, glucose was retained but fructose and mannose passed through completely. Galactose was partially retained; the explanation of this was found to lie in the fact that this sugar, when dissolved in water, splits up into α and β forms which differ only in the relative positions of an H and OH group attached to an asymmetric carbon atom; the α form was retained, while the β form passed through. Two further points are worth recalling. Glucose was retained when perfused in the amount which is normally present in the frog's blood plasma; but, when it was administered in larger amount, the glomerular membrane became permeable. Again, when a minute trace of a foreign substance (0.004 per cent. of phloridzin) was added to the normal amount of glucose, the membrane at once allowed the glucose to pass through; after removing the phloridzin with pure Ringer's solution, the membrane was restored to the normal condition, i.e. it was completely impermeable to glucose when perfused in the correct physiological amount.

Obviously, capillary endothelium is a very different sort of filter from glomerular epithelium, and experiments with the latter give no direct information about permeability from the circulation into the tissues or in the reverse direction. But they help one to realise that the mechanism of endothelial permeability must be extremely delicate, that it reacts selectively to minute stereo-chemical differences in the body fluids, and that the introduction of very slight amounts of foreign material may bring about a profound change.

With this introduction, I may formulate briefly the following ideas about the endothelial mechanism in relation to immunity.

¹ *Lancet*, II. pp. 1039-45. 1921.

378 *The Capillary Endothelium in Relation to Antibodies*

specific and non-specific factors in resistance to bacterial invasion. In entering upon these, it is necessary to discuss the nature of antibodies more closely and the part played by the capillary endothelium may assume a more definite importance.

The position bears some resemblance to the much discussed question about the nature of alexin. Nobody has succeeded in explaining what alexin really is. In ordinary laboratory routine this causes no difficulty; alexin, generally in the form of fresh guinea-pig serum, simply is "what it does"; and its capacity for doing certain work is very distinctive and can be estimated quantitatively with a high degree of accuracy. But some enquiring spirits, whilst accepting these facts, are not satisfied; they are not prepared to accept the inference that the work attributed to alexin is really due to the action of some special substance which, sooner or later, will be isolated by the biochemists and equipped with its chemical formula; they think that the action in question is attributable rather to the interplay of labile constituents in the serum and that all attempts to isolate and purify a special substance will lead to the destruction of "alexin" and will therefore defeat their own object.

Similarly with antibodies, one may start with the conception of a complex unit, of protein origin, which is chemically equipped so as to "fit" antigen. This is obviously true of the antibodies contained in an immune serum and may be demonstrated both *in vitro* and by appropriate animal experiment; and the amount of such antibody may be titrated with much precision. But is it safe to argue back from these serological data to the conditions which obtain in the living body of the naturally resistant or the actively immunised animal, and to postulate that in the latter circumstances the defensive mechanism consists, in part, of chemically distinct units identical with the special substances which are assumed to exist in an immune serum? Two questions have been raised about alexin. Does the work which is attributed to it in the test-tube correspond to work which is carried on in the living body? Is it a special substance? And about antibody, though differing from alexin in its greater stability *in vitro*, two similar questions may be asked. Does the living animal possess antibodies which are identical with serological antibodies? Is each different antibody reaction attributable to a different and special substance?

These questions involve various considerations, some of which are discussed in the following paragraphs.

Specificity of Antibodies.

Though it is convenient, and often quite legitimate, to distinguish "specific" from "non-specific" factors in immunity, it must be remembered that these terms are not always sharply separable and that neither is strictly appropriate to some of the events which take place during infection and resistance. For example, there is a colloidal balance between the fluids within the capillaries and the tissue fluids on the other side of the endothelium. When bacteria or

other foreign substances are introduced parenterally, this balance is disturbed and tends to be readjusted to a new level. The new level, in the adjustment of which the endothelium participates, is not "non-specific," since it differs according to the nature of the disturbing factor; on the other hand, one cannot go so far as to say that every different disturbing element tends to be followed by a correspondingly different (*i.e.* "specific") readjustment of colloidal equilibrium.

Similar considerations apply to the conception of antibodies as being either "specific" or "non-specific"; in some cases it may not be appropriate to use either term. The extremely delicate specificity of the precipitin reaction to foreign protein by no means justifies the assumption that there is a special antibody for every antigen, the former being "specific" when produced by immunisation with its particular antigen and "non-specific" when found in an animal which has not received this treatment. The normal constituents of plasma react in many ways to foreign protein which they encounter; and then, after this protein has produced some modification of the endothelium or other tissues, they tend to settle down to an equilibrium which may be somewhat different from their former equilibrium. In virtue of this change, when they again encounter this foreign protein their second reaction with it may differ, in some respect, from the first; but it is, in the main, the same type of reaction; the respect in which it differs does not justify a fundamental distinction between the reaction of an antibody (the second reaction) and the former reaction of the normal plasma, supplemented, where requisite, by the arbitrary postulate of normal or non-specific antibodies. In other words, what is currently known as an antigen-antibody reaction may be regarded as no more than a particular phase of a more general reaction between the constituents of plasma or serum and foreign protein.

According to this view, the conception of "antibodies" has to be widened. They are really much more than a particular chemical group (attached to a protein vehicle) which will "fit" a particular chemical group in the molecules of the antigen¹. It is also implied that they are of variable stability and that a distinction must be drawn between the highly unstable factors in immunity, which are inseparable from vital activity, and the relatively stable factors which may be demonstrated by serological tests. The real "antibodies" are the constituents of plasma as they exist in the living body; and their real mode of action is part and parcel of the ceaseless succession of interactions which is characteristic of living matter. In serum only remnants of them are left, *viz.* those remnants which have survived the changes from living to inert matter. It is suggested, further, that widening the range of "antibodies" in this way may be one means of satisfying the requirement for the discovery of "new kinds" of antibodies.

"Antibodies," then, are not mere counterparts of antigens, but are of a

¹ When using the word "antibody" in this wider sense, I write it with inverted commas, to distinguish it from the restricted sense of the term which is firmly fixed in immunological literature.

380 *The Capillary Endothelium in Relation to Antibodies*

quite different nature and are much more complex in their action. Their activities are those of a living mechanism and include the functions which are often (erroneously, in my opinion) attributed to special entities called complement or alexin. Antigens, on the other hand, are simply dead foreign protein, capable of being broken up in various ways; they certainly influence the living mechanism, but they are not an "active" part of it, in the sense in which circulating plasma or even fresh serum is "active." And this difference still holds good if, as I think is permissible, the conception of antigen is widened so as to include the varying products of metabolism which may occur in bacterial infections and may act antigenically, *i.e.* may lead to special (or specific) changes in the cells and fluids of the body. The real "antibodies" exist in the normal animal, whether resistant or susceptible, before the introduction of antigen; the antigen does not create them, though it modifies them in the case of the susceptible animal.

The conception of "antibodies" as a living mechanism in the circulating plasma differs from the purely chemical idea of them as molecules to which particular chemical groups are attached; this difference is involved in the view that in the change from life to death only the more stable functions of antibodies survive, *viz.* those functions which are not dependent on the ceaseless changes associated with metabolism.

The above ideas about antibodies and equilibrium are not new. I am not competent to correlate or compare them with Landsteiner's theory of electro-chemical affinities, but they seem to me to bear at least a rough resemblance to the following postulates which that author formulated many years ago¹. (1) An antibody may react with a large number of other substances which it encounters, the reaction with the homologous antigen being no more than a special or distinctive instance; it is superfluous to suppose that there is a chemical group of a particular configuration to correspond with each of the numerous reactions of an immune body. (2) The tissues of the normal, non-immunised animal have a capacity for uniting with colloids of widely different characters; this conception should replace the assumption that there are special receptors in each individual case. (3) An equilibrium is maintained between the colloidal components of one and the same animal body; and the disturbance of this condition through the introduction of a foreign colloid leads to new formation of immune bodies. (4) Antibodies form a series in an ascending scale of specificity. (5) Immune reactions are related to non-specific processes of adsorption.

This view also suggests a way of endeavouring to link up natural immunity with acquired immunity. Though there are obvious differences between the two, they have certainly much in common and it is highly improbable that they depend on entirely different mechanisms. It may, however, be a mistake to try to explain natural immunity in terms of acquired immunity. Why not try the reverse order of procedure?

In animals which are naturally immune against a particular bacterial infection, it is clear, at least in some cases, that antibacterial substances are present in the circulation, though, when the animal is bled, they are not usually demonstrable in the serum, even if this is revived or reactivated by the addition

¹ *Zeitschr. f. Immunitätsforsch. Orig.* ix, p. 779. 1911.

of alexin. Their disappearance does not justify the assumption that they are something quite different from "antibodies"; they may be labile "antibodies" which disappear, as such, on withdrawal from the living organism.

In the next place, the various species of animals which are susceptible cannot be lumped all together in sharp contrast with the naturally immune. On the contrary, the former all possess natural powers of resistance in greater or less degree and, in relation to a given type of bacterium, might be arranged roughly, according to their species, in a descending scale, commencing with the very highly resistant which are not far removed from the naturally immune, and progressing by easy stages to the very slightly resistant, in which high susceptibility is the more conspicuous feature.

In acquired active immunity, throughout the entire range of the susceptible groups, the main and primary fact may be regarded as a modification or reinforcement of the animal's natural antibacterial substances or true "antibodies," which, in the naturally immune animal, do not require such reinforcement. This change may or may not be followed by a secondary event, the appearance of serological antibodies. Their appearance, however interesting and important in other respects, should not cause one to lose sight of the primary event.

The Activities of Antibodies.

This view of the mechanism of "antibodies" may be developed a little further. Their activities may be regarded as presenting a variety of phases which should be considered one at a time.

(1) The normal constituents of the plasma are "foreign" in relation to any alien protein which is introduced parenterally. They may not react with it at all, as when the protein (or some of it) persists for a variable period in the circulation and is then excreted, unaltered, in the urine. But if, as is more commonly the case, they do react with it in one way or another, they are behaving as "antibodies." The most important example may be taken to be the way in which living bacteria are disposed of by the naturally immune animal. The normal plasma constituents interfere with the vital processes of the bacteria (by producing alterations in surface tension, in assimilation of food, in capacity for reproduction, or by other means) and their destruction, often completed by phagocytosis, is the result. This interference may be regarded as due to labile reactions between plasma and bacteria (or bacterial products) with the repeated occurrence of loose union followed by dissociation. In the end result, whilst the bacteria are disposed of, the plasma constituents have not been changed. This I regard as the primary and most important feature of "antibody" action. The "antibody" is in existence before the antigen is introduced and remains unaltered after the antigen has been disposed of.

(2) In the susceptible animal, the normal "antibodies" cannot accomplish this task unaided. Certain characteristic elements of the foreign protein (disintegrated bacteria or other non-living material) are adsorbed by the tissues,

382 *The Capillary Endothelium in Relation to Antibodies*

particularly by the endothelial cells. The consequent modification of the endothelial filter causes modification of the fluids which pass through it. Hence the plasma constituents become better adapted for forming loose union (followed by dissociation) with the foreign protein; when this protein is a living bacterium, they become better adapted to interfere with its vital mechanism. This modification in the properties of the original plasma constituents provides for the second aspect of "antibody" action; it is something which is "acquired," not as a new and independent mechanism but as a reinforcement of the natural mechanism. At the commencement of this phase the "antibodies" are still labile and are not demonstrable in the serum.

(3) In the next stage, the "antibodies," as they exist in the circulation, behave as before; their relations with antigen are still those of loose union and dissociation and do not result in a firm adsorption compound; but the change in the original constitution or balance of the plasma constituents is of a more permanent nature, with the result that the acquired affinity for the foreign protein survives in the serum and there becomes stabilised. This is the stage when the animal's serum may give a precipitin or other reaction *in vitro*, i.e. the stabilised antibody can now form a relatively firm adsorption compound with the antigen.

(4) If a disturbing factor is introduced, *viz.* reintroduction of the same antigen, there may be a partial reversion from stage (3) to (2). Is this to be explained on the ground that the labile "antibodies" are now given fresh work to do, in forming loose union and dissociation with the new antigen, and thereby lose some of their tendency to stabilisation, as is manifested by a temporary drop in the titre of serological antibody? This may be some part of the explanation, but probably the more important influence is the disturbance in the endothelial filter, caused by adsorption of new antigen, and followed by temporary increase in the instability of the "antibody" passing through into the circulation. The disturbance is only temporary and is soon followed by a return to stage (3), with perhaps increased output of antibodies demonstrable in the serum.

(5) After stage (3) has persisted for some time (up to the peak of the serological antibody curve), there is again a partial reversion to stage (2), without the introduction of any disturbing factor. This change is associated with the disappearance of the adsorbed antigen.

(6) Though the antigen has disappeared, the endothelial filter continues to turn out "antibodies" and these may be rendered less unstable (leading to a renewal of stage (3) with perhaps a still higher peak to the antibody curve) by the introduction of a non-specific influence. A good example of such an influence, according to Madsen, is manganese chloride, the assumption being, on my hypothesis, that, when this salt is adsorbed by endothelium, its catalytic action on the fluids which pass through diminishes their instability.

(7) The persistence of the capacity to form "antibodies" after the disappearance of the antigen varies according to the nature of the antigen; the

duration of immunity towards different infections ranges from a few weeks to a life-time. On what do these differences depend? On the capacity of certain cells (? endothelial filter) to renew, during metabolism and reproduction, that particular chemical structure which, by catalytic action, invests the filtered fluids with the properties of "antibodies."

(8) Continued production of "antibodies" over a long period means continuance of a modification (which may vary in degree) of filtered fluid. It is a qualitative conception, not quantitative, *i.e.* it does not mean that a certain quantum is manufactured on one day, a fresh quantum on the next day, and so on. Hence there is no need to imagine that the body is in danger of getting drenched with an "excess" of "antibodies," or that it has to save itself from this peril by constantly destroying them. On this view, there is no need to postulate that serological antibodies, even if they exist as such in the circulation of the actively immunised animal, are constantly undergoing destruction, in the way suggested by Madsen.

(9) The hypothesis that, in the change from living plasma to serum, "antibodies" lose their instability and assume the serological type does not exclude the assumption that there is also a tendency to this stabilisation in the living body, particularly when the "antibodies" are not freely circulating but become adsorbed to the surface of cells. Such adsorption and stabilisation, when it leads to the precipitin type of antibody, is the predisposing cause of anaphylactic shock.

Perhaps these considerations may help to explain some of the differences between reactions *in vivo* and *in vitro*. For example, serological tests may show that, in active immunity, apparently unaltered antigen may circulate for a long time in the living animal together with free antibody. Why does not neutralisation take place in the form of a precipitin reaction? It may, perhaps, be said that colloidal conditions in the living body differ from those in the test tube and are unfavourable for such a reaction; but this can hardly be the whole of the explanation. Though the antibody demonstrable in the serum was of the precipitin type, the "antibodies" actually circulating may not have been in a sufficiently stable condition to bring about this antigen-antibody reaction. There is a further point about the recovered antigen being apparently unaltered. Yes; unaltered in the sense that it is still a precipitinogen, but not necessarily unaltered in other respects by the circulating "antibodies." This last distinction may be of no particular interest when it is merely a question of identifying dead foreign protein by a specific reaction, but it may be of the greatest importance when the circulating "antibodies" are acting upon living bacterial protoplasm; they may be producing profound changes in the latter without destroying its "hall-mark" as specific antigen. Furthermore, one must accept the view that, in the course of immunisation, both antigen and antibody are constantly being adsorbed by fixed tissue cells, and must, therefore, frequently come into contact with each other in such situations. Then why is not immunisation an unfortunate reiteration of anaphylactic shocks?

384 *The Capillary Endothelium in Relation to Antibodies*

One reason probably is that the adsorbed "antibody" is not stabilised into the precipitin type.

So far, I have been considering "antibodies" in relation to active immunity. There are obvious differences in passive immunity, *i.e.* where the serological antibodies are transferred to a non-immunised animal. In such cases, the antibodies are already stabilised. Theoretically, they may act in one or more of three different ways: (1) they may remain temporarily in the stable condition, become adsorbed by bacteria or bacterial products, and enter into antigen-antibody combinations which are similar to those demonstrable *in vitro*; (2) they may break up into unstable "antibodies" behaving like those originally present in the circulation of the immunised animal; (3) they may be adsorbed by endothelium and modify the endothelial filter, thus acting like antigen specially prepared so as to produce immediate formation of "antibody." The possible importance of methods (2) and (3) lies in the fact that the efficacy of therapeutic sera, with the exception of antitoxic sera, cannot usually be explained by (1) alone; for example, the utility of anti-anthrax serum may be largely due to (2) or (3). And deficiency in these two properties may be the reason why many sera are of little therapeutic value though they are well provided with serological antibodies.

DOUBTS AND CONCLUSIONS.

Doubts.

Observed facts about the ways in which antigens and antibodies manifest their activities are generally recorded on the assumption that it is sufficient to define these substances in terms of what they are actually found to do. But sometimes it is desirable, in the interests of progress, to press for a closer definition and to ask for something more detailed than the statements that antigens produce antibodies and antibodies "fit" antigens. Then the trouble begins; one has now passed from the region of solid fact to that of tentative theory, where it is necessary to introduce physiological conceptions which are much vaguer than the concrete terms of chemical and physical reactions.

That is not the whole of the difficulty. If, in exploring a country, one comes to cross-roads, it is necessary to decide on the route to take. It may be possible to assure oneself that all the roads save one run in the wrong direction; then the one exception is clearly to be selected; it is the best working hypothesis. And if, at each point where the tracks diverge, one can come to an equally clear decision, there will be the satisfaction, at the journey's end, of knowing that every effort has been made to travel in the right direction. But if, at each of these successive cross-roads, it is impossible to exclude all routes but one, then the traveller has to admit, at the end of his journey, that his course has largely been determined by an element of chance or by his personal equation. He is open to the criticism that other persons, with equal justification (or lack of justification) would have mapped out quite different courses; and about one

and all of such explorers there will be added the remark that their exertions have been "merely speculative."

I can exemplify some of these "cross-roads" in the subject I have been discussing.

(1) It has often been said that knowledge of acquired immunity will not make rapid progress until more is known about natural immunity. Experimentally, the former kind of immunity is obviously easier to investigate, but it leaves the latter unexplained. One may start, however, with the view that acquired immunity is simply a reinforcement or readjustment of the mechanism of natural immunity. This hypothesis appeals to me as probably containing a considerable element of truth, and I have endeavoured to follow it up. Another person might choose quite a different course. He might say that the stimulus of infection brings into play a new mechanism, a factor which supplements the normal machinery but differs from it and is of independent origin, and that, therefore, acquired immunity cannot be explained in terms of natural immunity. I am unable to prove that this line of thought is devoid of justification, though I have preferred the alternative route.

(2) If natural immunity holds the clue to acquired immunity, then, as acquired immunity appears to be largely an affair of antibodies, the mechanism of antibody production must pre-exist in the normal animal, whether susceptible or immune. The adoption of this view leads, as I have endeavoured to show, to a wider conception of "antibodies." But there are alternatives which cannot be lightly dismissed. For example, it has not actually been proved that antibodies, of one kind or another, really are the main element in the normal defensive mechanism; it may be of quite a different nature.

(3) It is quite clear that the ordinary known serological antibodies do not suffice to account for either natural or acquired immunity (in their humoral aspects), and the distinction I have attempted between stable and unstable conditions of antibodies may help to account for this. But there are other possible explanations. Different kinds of antibodies may exist, though not yet discovered; or, again, it might be argued that the facts to be accounted for are not attributable to antibodies but to some other factors. For example, when the serum of a naturally immune animal is found not to be antibacterial, alternative hypotheses may be brought forward in place of the suggestion that the antibodies in the living plasma are unstable and perish in the serum.

(4) Endothelium has not been proved to be the site of antibody production. If, as is quite possible, this function really resides in some other types of cells, another explorer's route may diverge widely from mine.

I think the above examples are enough to make it clear that I have no illusions about the difficulty of pursuing the subject I have chosen with a coherent thread of argument.

Conclusions.

The most obvious feature about antigen-antibody reactions is their precision. They afford a constant reminder that the antibody must contain some special chemical component which exactly "fits" the antigen. But the number of possible antigens with which the animal body may have to deal, when foreign protein is introduced parenterally, is practically unlimited. This fact is illustrated in the text-books, which generally quote some colossal figure giving the number of different ways in which it is possible to combine the 20 amino-acids or "building stones" into which proteins can be broken up. It would seem, then, on the "lock and key" hypothesis, that there must be available in the animal body an equally colossal number of "keys." I see no particular reason to disagree with this line of thought. It is sufficient to point out that it leads to nothing; it merely implies that there is no cause for surprise, whatever may happen, all that has occurred being a particular rearrangement of "building stones."

The futility of attempting to identify innumerable different arrangements of "building stones" makes one realise that, in studying antibodies, one cannot be content to regard them as mere counterparts of antigens. To modify the metaphor and call antibodies "master keys" may be welcomed as a slight departure from the "counterpart" idea, but I do not think it leads very far. The main defect of the "lock and key" conception is that it gives no clue to the method and order which regulate the body's activities. That is what the immunologist wants—a physiological explanation of the way in which the animal organism produces antibodies. When he has obtained that, it may be taken for granted, without the necessity for chemical analysis, that the "keys" or "building stones" are arranged as they should be.

By a physiological explanation I do not mean the simple device of describing antibodies as enzymes which are secreted by cells of the body. That is often merely a change in nomenclature which is no more explanatory than the "lock and key" idea. Each antigenic variant of foreign protein stimulates certain cells of the body to produce a different and special kind of enzyme which acts as an antibody; this implies that the body is capable of producing an indefinitely large number of different ferments. As antibodies bear some resemblance to enzymes, one might give a qualified assent to this statement. But what is it going to lead to? To call antibodies "enzymes" does not produce order out of chaos; it merely postulates that a certain physiological function may be exercised in an infinite variety of ways. What is wanted is some idea of an orderly mechanism of antibody formation; when that is acquired, the minor question of a resemblance to enzymes may be discussed at leisure.

It is known that, as a result of immunisation, something is present in the serum which was not there before; and it is safe to conclude that the serological change is referable to some event which has occurred in the living body. But one cannot make the converse assumption that changes occurring *in vivo*

during immunisation are necessarily represented by demonstrable changes in the serum. On the contrary, as there are many events in immunity and infection not associated with the presence or absence of serological antibodies, it seems more natural to assume that some of the properties of the circulating antibodies have been changed or lost in the transition from living plasma to inert serum. And, unless cause can be shown for ruling it out of court, I think this latter assumption should be tested as a working hypothesis in the physiological explanation of antibodies.

How does the foreign protein act as a stimulus? Various experimental data lead one to think that the first event is adsorption of some of the protein constituents by the surface of certain tissue cells. But what is the next step? That is the difficulty. Definite proof is lacking and it is necessary to select from alternative hypotheses. Is it to be said that the adsorbed protein causes the cell to become a special kind of chemical laboratory which proceeds to turn out antibody? Before agreeing that this is the best explanation available, I think it is worth considering whether filtration may not play some part in the process. It seems to me that this is plausible, particularly if one supposes that the capillary endothelium acts as the filter.

With reference to the earlier stages of antibody formation, two hypotheses which I have put forward are (1) that the properties of the endothelial filter are changed by the adsorption of foreign protein and (2) that antibodies are formed by the modification of the fluids which pass through this altered filter. But antibodies may still be turned out at a later stage, when the adsorbed foreign protein has disappeared. To account for this, a further hypothesis is necessary, (3) a persistence, in the normal constituents of the endothelium, of some impression which was formed in the earlier stages. The result of the "impression" is persistence (for a period of variable duration) of the modified character of the filter; the nature of the "impression" is assumed to be some alteration in normal metabolic processes, an alteration which may be passed on to new generations of cells.

The above are examples of the working hypotheses which I have attempted to develop in the preceding pages. I do not claim that they are necessarily the best; out of the many possible alternatives, probably other persons who may be interested in this subject will find that a more helpful selection might have been made. My main contention is that more attention should be paid to the development of a physiological conception of antibodies.

VEGETABLE DECOMPOSITION IN DITCH WATER SIMULATING SEWAGE CONTAMINATION.

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(With Plate V.)

LATE in the autumn of 1922, following an exceptionally dry period, the water of some of the ditches in the "Backs" at Cambridge became foul, so that instead of looking dark and clear, as it usually does, it became milky in appearance, and gave off an offensive odour so like that of sewage that many people believed that it had become contaminated by leakage from the sewer near by. This led to an investigation, which proved that no pollution with sewage was taking place, and that the trouble was due solely to vegetable decomposition, aided by stagnation of the water, the result of an unusually dry season.

Since the possibility that decay of leaves in stagnant water may simulate contamination with sewage does not seem to be widely recognised, and since such an occurrence is liable to alarm people unnecessarily, and, perhaps, to lead to useless expense, it seemed desirable to record this experience. Consequently in the following paper there is given a short account of the state of the ditches during the period when they attracted attention on account of their objectionable state, and of the bacteriological observations which were made with the water.

* * * * *

Attention began to be directed to the ditches about the end of October, 1922, when the odour emitted by the water became the subject of much complaint. It resembled, indeed, very closely that of sewage. Hydrogen disulphide was clearly distinguishable. This gas may, of course, be detected whenever the mud at the bottom of any ditch or pond is disturbed, but here it was evident without any disturbance of the mud, and to it were added other odours which give to the smell of sewage its distinctive characters.

The appearance, also, of the water lent support to the theory of sewage contamination. Bubbles occasionally appeared on its surface; but these were, on the whole, not much in evidence. A much more striking change was pronounced milkiness, which would appear from time to time like a cloud in the water, now in one place, now in another, and, after lasting for a week or more in any one region, would slowly fade away to appear elsewhere. This appearance was very striking, and it is scarcely an exaggeration to say that, at its height, the previously clear black water looked as if a can of milk had been

emptied into it. It came and went, as has already been said, and the sewage-like odour varied with the milkiness.

The low-lying land on the west side of the river Cam where it flows past some of the colleges from Queens' to St John's, is divided up into a number of paddocks, each of which is surrounded, in the seventeenth century Dutch manner, by ditches, similar to those seen in the well-known picture by Hobema, in the National Gallery. The ditches open, for the most part, directly into the river. There is seldom any perceptible flow of water in them, but at ordinary times they are clean, and filled with water which is clear, or, at worst, covered with duck weed. They are overhung with trees—limes, elms and chestnuts; and in the autumn they get filled with leaves from these trees and the many others around them.

On the far side of the paddocks, running parallel to the river, is the Queen's road, under which is a deep sewer. On the further side of the road the ground, which here rises a little, was, before the war, occupied by college gardens and cricket fields. But on one of the latter, there grew up the First Eastern, Military Hospital, and, its buildings are now temporarily occupied by a civil population of 200 families. The change in the water was first observed in the ditches which lie at right angles to the river, on either side of Garret Hostel Lane, and especially at their ends furthest from the river, and nearest the Queen's Road, where the drain from these dwellings joins the main sewer under that road. It was therefore not unnatural to suspect that a leak had occurred in the sewer at this point, especially as no one remembered to have seen the ditches in such a state before, and the exceptionally dry summers¹ were known to have caused shrinkage of the gault and subsidence of the foundations of some of the houses in the neighbourhood; and it was thought that the shrinkage might have affected the sewer also².

Thus it was not without cause that sewage contamination was suspected, in spite of official denial. I confess that I myself held this view, until convinced by personal investigation of its untenability, although the Borough Surveyor assured me that the sewer was intact, and that even if there were a leak the sewage which escaped could not possibly get into the ditches owing to the greater depth of the sewer.

And there were other factors, undervalued by me at the time, which did not fit in with the theory of sewage pollution. For already in the autumn of

¹ The summer of 1921 was so exceptionally hot and dry that it will not easily be forgotten; wells and streams around Cambridge dried up, and the lack of water in country districts became severe. The winter which followed did not make up the deficiency, and was followed by a hot and dry spring and early summer. There was, it is true, a good deal of rain in July 1922, but less in amount in Cambridgeshire than in most other parts of the country. It was not sufficient to fill up our wells, and after the very dry autumn which followed, the shortage of water in Cambridgeshire was worse than ever. A countryman told me that his well, which had held out through 1921, had become dry in the autumn of 1922.

² The sewer, however, at this point does not lie in the gault, but in a bed of sand and gravel of considerable thickness overlying the gault.

1921, the ditches in question had attracted unfavourable notice for the same reasons as drew so much attention to them a year later. But the trouble did not then attain to such proportions as it did afterwards, and no great complaint was made. The Colleges concerned were, however, requested to clean out their ditches, which they accordingly did, and the water remained clean during the following summer.

Thus the trouble was recurrent; it occurred each time, after the fall of the leaves in the early winter, and not in the summer when sewage contamination, if it took place, might be expected to cause most offence. It ceased after the ditches had been cleaned out.

Investigation was first made of the extent and distribution of the trouble, and directed to finding its points of greatest intensity, if any. The local condition of the water was plotted on a six inch map, and the variations which occurred from time to time were recorded.

The ditches out in the open country around Cambridge were examined and found nowhere to be in a state similar to those in the "Backs," even when they were full of leaves¹. In the "Backs" themselves the ditches were not all equally affected; and it was the fact that the maximum pollution seemed to coincide with the points most likely to be reached by sewage escaping from the drain in the Queens' road that lent such probability to the view that the nuisance was traceable to the sewer.

But, on the other hand, there was a ditch just outside the town, namely on Coe Fen and bordering the Leys School grounds, which was even more affected than the Garret Hostel ditches; and there did not seem any possibility that this ditch could have been polluted with sewage.

Such was the ambiguous state of the investigation when, in the middle of December, the Commissioners of the Cam, at the request of the Mayor, kindly consented to lower the level of the river, so that the ditches might be emptied of their water. While they remained in this condition, for a period of nearly a week, they were carefully inspected by the Medical Officer of Health, Dr Laird, and myself to see if any signs of sewage running into them could be detected. We had already determined, as I have said, on the most likely spots, and these were subjected to a thorough examination. But no trace of sewage could be detected.

When the river was allowed to rise again to its normal level the ditches became filled with clean water once more; and copious rain falling about this time, some movement was re-established in them. They have since then remained in a satisfactory condition².

Thus the result of this investigation was to convince us that no leakage of sewage into the ditches was taking place, and that the cause of their foul condition must be looked for in other directions. It remained to be seen whether

¹ Since this was written, a ditch far away in the country and which could not possibly contain sewage from any human habitation was found in a similar condition. This was in July 1923.

² A very slight return of the conditions described was noticed by me in the summer of 1923.

the bacteriological investigation which was then in process would reveal the cause of the unusual condition of the water.

Bacteriological Investigation

Samples of the water were taken in sterile Winchester quart bottles from the ditches on various occasions and removed to the laboratory.

An attempt was first made to determine whether the milkiness of the water was due to a cloud of bacteria, or to chemical matter in a fine state of division suspended in it. Samples put up and examined in the hanging drop under a $1/12$ th oil-immersion lens showed numerous bacteria, but it was difficult to decide whether these were present in numbers sufficient to account for the appearance. Sterile tap water, contained in one-litre flasks, and to which a tube or two (about 10 c.c.) of nutrient broth had been added, became, when sown with some of the milky water and incubated for a day or two, as cloudy as the ditch water itself. And so also did similar flasks sown with certain cultures which were isolated from the ditch water, and which I shall presently describe. After due consideration I am inclined to the view that the turbidity of the ditch water was directly due to the cloud of bacteria in it, and not to finely divided non-living matter in suspension. The cloudiness did not disappear on the addition of acid.

Plate cultures on ordinary nutrient agar yielded various bacteria. It was significant that *B. coli* was not recognised among them. Two mono-flagellate, round ended, short bacilli, however, at once attracted attention on account of the characteristic odour produced by their cultures. These became known in the laboratory as S. 1 and S. 2, and under these names will be described here.

Two Bacilli isolated from the Water of the Ditches, and capable of causing Foul and Characteristic Odours.

These two bacilli have many properties in common: They produce a copious, confluent growth on various kinds of media, including McConkey's bile-salt agar; they are Gram-negative, stain rather feebly with various dyes, and form acid but no appreciable amount of gas; they are actively motile, and each kind is provided with long, single, terminal flagella. They grow at various temperatures from 3° C. to nearly 40° C. Perhaps their most notable feature is their variability of form which, under favourable conditions of temperature may be an oval bacillus, and at higher temperatures long curved rods and undulating threads.

While the most important characters of these bacilli have thus been briefly recorded it will no doubt be desirable to give in addition a more detailed description of them, and to refer particularly to the points wherein S. 1 differs from S. 2.

Cultural Characters.

Growth of either species is plentiful and fairly rapid at all temperatures from 9° C. to about 34 or 35° C. At 3° C. it is slow, does not appear for some days, and never becomes luxuriant. As the temperature approaches the opposite limit growth again becomes impaired; at 37° C. it is distinctly scanty, and it ceases altogether before 40° C. is reached.

On nutrient agar, from 9° C. to 34° C. each species forms a copious, moist, pearly grey, translucent growth, very like that formed by *Vibrio cholerae* or one of the bacilli included in the typhoid-coli group. The growths of S. 1 and S. 2 are not precisely alike; often there is very little difference between them, but on the whole that of S. 1 tends to become a shade thicker and whiter than that of S. 2.

This difference, slight though it is, comes out better in agar plate cultures, when separate colonies are given plenty of room to develop. Under these conditions S. 1 grows in rather large thick whitish colonies which, when there are not more than 20 or 30 to the Petri dish, may be as much as 5 or 6 mm. in diameter. S. 2 under the same circumstances produces slightly smaller and thinner colonies. Thus if, in respect to their colonies on agar, S. 1 may

be compared with *B. coli*, S. 2 may be said to resemble rather *B. typhosus*. Both are moist, and free from stickiness as a rule, but when grown at 37° C., and therefore producing long threads (which one imagines may possibly become entangled together) they are somewhat mucoid; and old cultures of S. 2, even when grown in the cold, show a tendency to draw out into a thread when the platinum needle which has touched them is being withdrawn. It is tempting to connect this with their habit of forming capsules, indications of which have been observed and will be described in a moment.

On serum growth is less copious than on agar.

On potato it is luxuriant, a thick pale cream coloured film being produced in 24 hours.

In broth growth appears as a cloud which is appreciably denser in the upper layers, and, in the early stage, a ring of denser coherent growth may occur where the surface of the fluid is in contact with the walls of the tube—a ring similar to one which I have often seen in cultures of *V. cholerae*.

In gelatin-shake cultures of either bacillus growth is confined for the most part to the surface and upper layers of the medium. Colonies in the substance and visible to the naked eye are practically confined to the upper half centimetre and rapidly fall off in size the deeper they penetrate. At first sight the rest of the gelatin seems to be free from growth, but close inspection in a good light, and particularly with the aid of a hand lens, reveals minute colonies extending to the bottom of the tube. This is a constant phenomenon and repeats itself when the cultures are sown from single colonies. It is rather more obvious with S. 1 than with S. 2.

No gas bubbles appear. There is, of course, the usual disagreeable odour, and therefore presumably gas, but, obviously, it is not produced fast enough to become entangled in the gelatin and to collect into bubbles.

The difference in the surface colonies of S. 1 and S. 2 on gelatin, again as on agar, are best seen in plate cultures. Growth is slow and the colonies continue to increase in size for several days. Those of S. 1 become, when moist, strongly raised and of a pearly white colour, reminding one of the hemispherical colonies of the bacillus of Friedländer. At their best they attain a diameter of 4 or 5 cm. When old and dried they may have a raised centre and a thinner indented margin. Those of S. 2 appear as white spots of smaller size lying in the centre and at the bottom of cup-shaped depressions in the gelatin. These depressions are sharply defined and circular. They contain little or no fluid, the liquefied gelatin being, apparently, absorbed by the surrounding jelly.

The deep colonies of S. 1 are not distinguishable from those of S. 2, or at most are only very slightly larger and whiter; both are lenticular in shape and small in comparison with the colonies on the surface.

In gelatin stab cultures S. 1 forms a large raised white colony at the surface, S. 2 a smaller colony, thinner and greyer. Down the track of the needle, there is growth only for a few millimetres after which the visible track fades rapidly away.

There is a very slight greenish fluorescence seen sometimes in the upper part of cultures of S. 1 but not in those of S. 2.

The liquefaction of S. 2 begins at the surface and proceeds but slowly. After 24 hours a little cup-shaped hollow is just visible, and next day the appearance is rather like that of a typical cholera culture. After a week or 14 days liquefaction has only extended about a centimetre or two from the surface.

In 1 per cent. peptone water both species grew well, and if glucose be added they produce acid but no gas. The fluid in the Durham's tubes, inserted to catch gas should any be formed, retains its blue colour for days, showing that the glucose there is not fermented, and that the microbes will not grow under the anaerobic conditions which prevail there. When lactose is the only sugar present no acid is formed, but the medium, to which litmus has been added, may become bleached in its deeper part.

It seems probable that these bacilli prefer weak solutions of organic matter to strong ones as culture media. In water to which only one or two per cent. of nutrient broth, or the same quantity of the boiled meat extract used for making the broth, had been added they grew well, and produced a marked opalescence or turbidity comparable to that of the ditch water seen under similar conditions of bulk and light. In gelatin (10 per cent.) great reduction

of the amount of nutrient broth added did not seem to make any difference to the amount of growth.

On McConkey's bile-salt agar these microbes grow freely—in which point again they resemble *V. cholerae*.

Microscopic Characters.

One of the most notable characters possessed by these bacilli is their variability in size and shape on different media and especially at different temperature. It will therefore be necessary to describe their microscopic characters rather closely.

But not only do these bacilli vary under the conditions named above but their length and appearance is not quite constant on any given medium or at any given temperature. This seemed to be due to slight and uncontrolled variations in different batches of the same kind of medium. Thus one set of potatoes would grow longer bacilli than others. The same was true of different batches of serum or even of agar, and it seemed to be generally the case that when the growth was scanty the bacilli were longer, and when it was copious they were shorter, as though qualities in the medium favourable to massive growth promoted also rapid cell division, so that long individual bacilli tended to disappear. We shall see that when the temperature of cultivation is raised to 37°, at which level growth is always scanty, much longer forms are the rule. On account of this variability the measurements given below must be taken as approximate only, and the descriptions as applicable to average specimens.

On ordinary nutrient agar at 10° C. or 23° C. both species appear, for the most part, as oval bodies, separated from one another by rather wide intervals. They measure 1.5 to 2 μ in length and are about half as broad as they are long. A few longer forms may appear. At unfavourably low temperatures, c. 3° C., S. 1 grows in distinctly longer forms. They both stain feebly with methylene blue, or even with methyl violet, unless the staining is prolonged, and they usually appear as a dark outline with pale interior. A few longer forms, as already mentioned, may be seen. These are apt to stain more darkly.

Occasionally there may even be a thread (commoner in S. 2 than in S. 1) such as one sees in cultures of *B. typhosus*, and foreshadowing what may occur at higher temperatures.

With carbol-fuchsin they show polar staining, and in longer forms, such as occur in cultures about to be described, the stainable material is broken up by the violence of the stain into a number of segments¹. Such appearances have probably no relation to the normal structure of the bacillus.

On some potatoes they grow, at 22° C., as definite bacilli three or four times as long as they are broad, and 3 or 4 μ in length, round ended and somewhat curved, and only occasionally arranged end to end. On other potatoes especially when growth is very luxuriant they are shorter and approximate to those seen on agar cultures grown at similar temperatures.

The bacilli grown on broth or serum are similar to those grown on potato, and are, on serum especially, equally variable.

When the temperature of incubation is raised to 30° C. the bacilli, even on agar, grow longer and approximate to those grown on potato. On raising the temperature, very little change takes place until the region of 37° C. is reached. At that temperature, when growth is as we have said, scanty, the bacilli, after 24 hours' incubation, are long curved rods 8 or 10 μ in length, and if the temperature of incubation be approximated to 40° C. involution forms appear, long undulating threads, and sometimes strings of ill-shaped sausage-like bacilli, or threads swollen and staining more feebly than the others and with little sparsely scattered darkly stained granules adhering to their cell membranes.

These descriptions all refer to 24 hour cultures at the temperatures mentioned. When grown longer no important changes were observed. No polar bodies or spores were seen at any time. The bacilli, in spite of the absence of spores, remain alive when left in the cupboard for many months.

In some of the microscopic preparation, especially when stained darkly with methyl violet, there was a definite suggestion of capsules (see Fig. 2).

¹ A similar appearance in *B. coli* and *B. typhosus* may be seen in Figs. 56 and 61 in Mallory and Wright's *Pathological Technique*. It is not stated what stain was used.

The flagella, stained by Stephen's modification of Van Ermengem's method, were single and approximately terminal. They showed, for the most part, three or two and a half complete undulations, and appeared considerably longer than those of *V. cholerae* and more like those of *B. pyocyaneus* except that the undulations were more pronounced.

Taking all their characters into consideration these bacilli seem to be akin to *V. cholerae*; and from their Gram-negativeness, their incapacity to form an appreciable amount of gas from various sugars, their habit of forming under certain circumstances long undulating threads, and more particularly, from their possession of single terminal flagella they may, I think, be regarded as belonging to the family of spirilla.

S. 1 produces in artificial cultures an unpleasant odour, not unlike that of *B. coli*. S. 2 produces a more offensive smell in which a suggestion of putrefaction is combined with the smell of over-ripe quinces. Both are capable of producing a cloud in water to which a very small amount of organic matter (e.g. 1 per cent. of boiled meat infusion) has been added.

For these reasons I believe that the bacilli in question, but especially S. 2, were mainly responsible for the abnormal milkiness of, and the offensive odour emitted by, the water in the ditches.

Probably other micro-organisms contributed as for example by producing H_2S . No such micro-organisms were isolated from the ditches; but much remains to be done with investigation of the anaerobic bacteria present in the water.

Chromogenic Micro-organisms.

Some chromogenic micro-organisms found in the water, and probably the cause of certain appearances of colour in the contents of the ditches which I have not previously alluded to, deserve a few words of description.

Shortly before the water was drawn off from the ditches my attention was called to certain patches of pinkish or violet colour which were making their appearance here and there on the leaves and twigs lying at the bottom of the water, and on the boards which line the sides of the ditch on the East of St John's Wilderness. This colour became still more evident on the wood when the water was drained away from the ditch. It was obviously caused by a thin layer of some alga-like growth. Samples were collected of the more brightly coloured of the leaves and sticks, brought to the laboratory, and put, together with ditch water, in large covered glass dishes. In the course of a day or two a violet coloured micro-organism was found to be growing in indefinite patches, an inch or more wide, at the bottom of one of the glass dishes. Numerous efforts were made to get this to grow in pure culture, but all failed, although, among others, a medium made of a decoction of leaves and ditch water was employed.

The micro-organism was at first thought to be *B. violaceus*, but though I had no difficulty in cultivating this latter micro-organism from the ditch water, that which we saw growing in the coloured patches was of another order.

Under high magnification it was found to consist of little curved, round-ended, sausage-shaped bodies, on some of which could be seen a single wavy flagellum. They were about as long as the diameter of a red blood corpuscle, and probably almost as deeply coloured, for their colour was quite apparent even when seen under the oil-immersion lens. They appeared to have no nuclei or spores, unless indeed little colourless highly refractile bodies of which several, or in some, many, could be seen in each cell, were of this nature. Sometimes

the coloured contents of the cell did not extend quite to its rounded extremities, as if it had shrunk away a little from the envelope. Occasionally two cells were seen end to end. These flagellates were found, not only when growing on the glass bottoms of our dishes, but on the coloured leaves and twigs, and there can be little doubt that they were the chief, if not the sole, cause of the strange appearances of colour noticed in the ditches.

I do not think that *B. violaceus* played any part in these appearances, though, as already said, it was readily isolated from the water in pure culture.

Apart from the coloured patches of growth just referred to, the water itself, sometimes and in certain places, seemed to be tinged with a similar violet-pink colour, and on one occasion, in the laboratory, a litre flask of unsterilised ditch water to which had been added some meat infusion, and which had been sown with S. 1 and S. 2, turned distinctly violet; but I was unable to isolate any coloured bacteria from it; nor did a flask of similar water turn violet after being sown with *B. violaceus*.

SUMMARY AND CONCLUSIONS.

In autumn and early winter certain ditches around Cambridge became offensive, and their water turned milky. This led to a grave suspicion of sewage contamination, which, however, was satisfactorily disproved. On the other hand certain bacilli with single flagella were isolated from the water of the ditches and found capable of giving off from their artificial cultures an odour comparable to that of the ditches. And to these bacilli, acting on the dead leaves which found their way into the water, and, probably, in conjunction with other anaerobic bacteria which were generating hydrogen disulphide, the sewage-like odour is attributed.

Certain patchy changes of colour in the leaves and wood immersed in the water are attributed to a chromogenic flagellate which was found growing on them.

DESCRIPTION OF PLATE V.

Fig. 1. S. 2, from an agar culture, stained by Stephen's modification of van Ermengem's silver nitrate method. S. 1 is similar.

Fig. 2. S. 1, from a 24 hour growth on agar at 22° C. stained darkly with methyl-violet, showing indications of capsules.

Fig. 3. S. 1, from a 24 hour growth on agar at 36° C. stained darkly with methyl-violet. The granules are probably artifacts caused by overstaining; they were not seen in lightly stained specimens.

NOTE. My thanks are due to Dr M. R. Swann, for kindly taking the photographs that are reproduced in this plate.

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Fig. 1

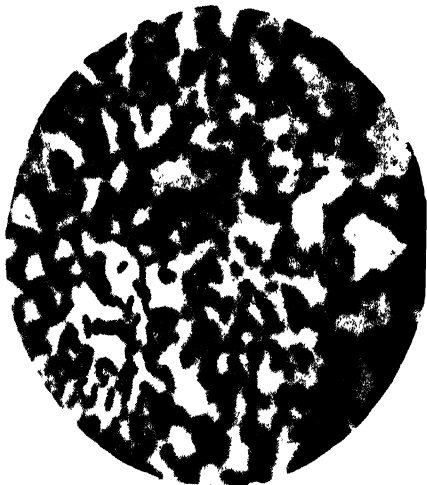


Fig. 2



Fig. 3

A COMMENTARY ON RECENT PLAGUE INVESTIGATIONS IN TRANSBAIKALIA AND SOUTHERN RUSSIA.

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ALTHOUGH much work on the epidemiology of plague has been done in many parts of the world since the discovery of the *Bacillus pestis* in 1894, the origin of the outbreaks in Eastern and Southern Russia has, until a short time ago, remained obscure. Transbaikalia, together with extensive areas of Northern Manchuria and North-east Mongolia that are conterminous with it, and, again, the region in Southern Russia which includes the Kirghese and Kalmuck steppes and especially that portion of it which lies between the lower reaches of the rivers Volga and Ural have long been known to contain endemic foci of plague, and have been the source of considerable outbreaks of pneumonic plague. Thus, in the winter of 1878-79, an outbreak of this type at Vetlianka, a Cossack village on the right bank of the Volga, caused alarm in Western Europe. Competent epidemiologists—British, French and German—visited the village after the event, and examined the circumstances that favoured the spread of the infection. Their observations were brought together and analysed by Netten Radcliffe (1881) in his memorandum on plague, which gives the first adequate description of a pneumonic plague epidemic. The more recent epidemics of pneumonic plague, namely, those of Manchuria in 1910-11 with 50,000 deaths, Middle China in 1917-18 with 15,000 deaths, and Manchuria in 1920-21 with 9000 deaths, owed their origin to ill-defined centres of infection in the immense tract of land which includes Transbaikalia and which is contiguous to the north-west boundary of China.

ENDEMIC PLAGUE IN TRANSBAIKALIA.

China has lain under the menace of serious outbreaks of pneumonic plague ever since the great epidemic that lasted throughout the winter of 1910-11 in Manchuria. When this epidemic was at its height, the Chinese Government invited the eleven foreign powers represented at Peking to send to the seat of the outbreak investigators who possessed experience of plague so that they might devise measures for the prevention or control of future epidemics. The delegates to the International Plague Conference held at Mukden in April, 1911, and the witnesses who gave evidence before it did valuable service by providing a carefully documented report of all the aspects of the epidemic. The recommendations drawn up for the guidance of the Chinese Government have doubtless been useful in helping to limit the later outbreaks.

The opinions of the delegates to the Conference upon the source of the outbreak of 1910-11, as expressed in their report, are as follows:

(1) The past epidemic arose in a region which has been associated for years with outbreaks of pneumonic and bubonic plague, but sufficient evidence is not forthcoming with regard to its precise origin.

(2) From Russian medical sources it has been reported that an epizootic disease exists among tarbagans and that it is not unlikely that this disease is plague, but that it is plague has never yet been proved bacteriologically.

(3) There is no definite evidence to show that the first cases of this epidemic were caused by infection from sick tarbagans. Nevertheless, there is strong presumption for believing that tarbagan disease is closely associated with pneumonic plague in Manchuria, Transbaikalia, and north-east Mongolia, and, therefore, with the recent outbreak.

During the proceedings of the Conference two communications were read (on April 16, 1911) which came near, as events have shown, to the heart of the problem. In one of them Strong, the chief delegate of America, brought forward experimental proof of the susceptibility of tarbagans to acute plague. The other, by the present writer, gave a brief account of the flea infestation of twelve tarbagans he had the opportunity of examining; discussed its bearing upon the spread of plague amongst tarbagans and the transference of the infection from them to man; and noted that, if a secondary pneumonia should supervene in a patient who was thus infected, the contacts would be exposed to the risk of contracting primary pneumonic plague. The contributions of Strong and of myself give, in effect, a forecast of the direction in which investigations have proceeded and of knowledge that has been gained. Recent work, of which a summary follows, makes it clear that bubonic and pneumonic plague in Transbaikalia and the adjoining regions is traceable to epizootic plague in tarbagans.

In June, 1911, soon after the Conference came to an end, Zabolotny and Tchurilina demonstrated for the first time spontaneous plague-infection in a tarbagan which was found by Issaew near Borsja station in Transbaikalia, 80 miles west of the Manchurian frontier. Later, Pissemsky ascertained that bubonic plague was prevalent amongst tarbagans near Araboulak and Lake Tschaborda in the same district. Wu Lien Teh (1913, 1922) thought that the tarbagan was not an important distributor of the infection, but while visiting in June, 1923, the Russian Plague Laboratory at Sektui¹, a village in Transbaikalia about 30 miles from the Manchurian frontier, he had an opportunity of observing plague in tarbagans found in the neighbourhood, and now recognises that tarbagan plague is the origin of the epidemics in China.

¹ Bieliavski (1895) gave an account of an outbreak of plague in September 1894 in Sektui. "The origin of this outbreak of plague was easily traced. The first patient had just before his illness gone to attend a court at Tzagan-Olui. On the way there his dog caught and killed, in a very brief time, six tarbagans, which the man carried some versts, and then hid in some straw by the roadside, to pick them up on his return. He returned on August 31, and was taken ill on September 2. The rapidity with which the animals were caught seemed to show that they must have been suffering from disease." (Quoted from Clemow, "Plague in Siberia and Mongolia, and the Tarbagan," *Journ. of Trop. Med.* 1900, p. 170.)

A recent paper by Jettmar (1923), whose headquarters are at Chita, a town in Transbaikalia not far from a tarbagan-infected area, gives useful information on the subject. He states that in this area the tarbagan is the only steppe-rodent in which plague infection exists. Rats (*R. norvegicus*) frequent only a few of the houses in the Cossack villages; they do not come into close contact with the tarbagan; and they play no part at all in the origin of human plague in Transbaikalia. Although other species of steppe-rodents are more or less susceptible to plague, a natural infection has not been discovered in them. The first human cases in the tarbagan-infected districts are met with almost invariably in the late summer and in the autumn; they are always of the bubonic form. Primary pneumonic plague has its starting-point in one of the contacts of a patient who is suffering from bubonic plague and in whom a secondary pneumonia has supervened. These statements are confirmed by the assertion of Zabolotny (1923) that during the last epidemic of pneumonic plague in Manchuria (1920-21) plague was epizootic amongst tarbagans in Transbaikalia; that pathological and bacteriological proof of the nature of the infection was obtained; and that the epizootic preceded the human cases: tarbagan hunters were the first to be attacked.

Jettmar notes that the tarbagan flea (*Ceratophyllus silantiewi* Wagner) can imbibe human blood; its ability to bite man seems to be widely recognised by the hunters and the steppe dwellers. Young tarbagans harbour considerable numbers of fleas, up to nearly a hundred on one animal. His observation that the favourite situation of the fleas is the fur of the neck, and the fact that cervical buboes are commoner than axillary and inguinal buboes give support to the belief that flea transmission is the normal mode of infection amongst tarbagans; they recall similar observations in India by the Plague Research Commission on naturally infected rats and on rats and guinea-pigs that were experimentally infected by means of fleas. In the human bubonic cases in Transbaikalia axillary buboes are seen nearly as often as inguinal buboes; a proportion which is higher than that found in plague derived from rats and which is comparable with the preponderance of axillary buboes in the persons who were infected from the ground-squirrel in California, an animal which, like the tarbagan, is hunted and used as food. Jettmar furnishes a detailed list of the ecto-parasites of the various steppe-rodents in Transbaikalia.

The literature dealing with the tarbagan and its relation to human plague has an added interest in view of the knowledge we now possess; the earliest reference is to the observations of Tscherkassow in the year 1857 in Eastern Siberia. The following sources of information cover most of the ground: (1) Sticker's monograph on plague (1908, 1910), (2) Clemow's useful abstract (1900) of the first important papers on the subject, which were published in 1895 by Bieliavski and Rieshetnikof respectively, and (3) a paper by Dudchenko (1909)¹.

¹ I was able to obtain a translation of Dudchenko's paper through the courtesy of Prof. Zabolotny and Dr Paul Haffkine.

In reviewing these publications one is impressed by the consistency of the narratives and by the exactitude with which the details fit in with newly acquired knowledge. The inhabitants of the steppes—the Buriats, Mongols and Russian Cossacks—have long been acquainted with the danger of handling diseased tarbagans; and indeed their appreciation of the risk gives point in an interesting and unmistakeable fashion to the tarbagan legends that have been recorded—the one in the year 1856 by the naturalist Radde, who was the first to study the habits of the tarbagan, and the other in 1902 by Smolieff (cited by Sticker and Dudchenko). There has been no tendency on the part of the medical authorities to doubt the essential conjunction. Sticker, for example, who has an unequalled familiarity with the history of plague throughout the world, accepted on epidemiological grounds alone the tarbagan disease as plague and was convinced that it was the source of the human outbreaks. The circumstance that tarbagans constitute a natural reservoir of the infection cannot be regarded as singular, for it is paralleled by observations made in widely scattered localities where epizootic plague in a variety of field rodents has given rise to outbreaks of bubonic and pneumonic plague in man (California, Orange Free State, Suffolk in England, Persia, and Southern Russia).

ENDEMIC PLAGUE IN SOUTHERN RUSSIA.

It is good to learn that Professor Zabolotny, the chief delegate of Russia to the Mukden Conference, has been able during the past three years to organise and direct extensive investigations into the problem of the endemic focus in Southern Russia. A summary of his observations is given in the June number (1923) of the *Annales de l'Institut Pasteur*. He and his co-workers have obtained proof that the endemicity of plague in the steppes of south-west Russia is due to spontaneous epizootics amongst spermophiles (*S. musicus* and *S. rufescens*). Aristarkowa, Denisowa and others have shown that the spermophile flea can bite man. The epizootic lasts from May to August, with a maximum in June. The first human cases in the bubonic outbreaks were *gardes champêtres* (rural policemen) who had been in contact with spermophiles.

The autumn and winter epidemics of pneumonic plague in the Don and Ural districts bear no *direct* relation to the epizootic, because the spermophile begins to hibernate in August. Winter epidemics of plague in the Ural district originate from epizootics of plague in field mice, which make their way into the houses.

Zabolotny believes that the fleas of the steppe-rodents are the agents which spread the infection in the epidemics of bubonic plague; and that the evolution of mixed epidemics (bubonic and pneumonic) depends upon contact infection from bubonic cases that are complicated by a secondary pneumonia. His conclusions are in virtual agreement—so far as they are comparable—with those which the writer and Major Ronald E. Todd, R.A.M.C., have set forth in a comprehensive review of the epidemiology of pneumonic plague that

forms part of a report of work carried out on behalf of the Egyptian Government and that has recently been published in Cairo. In the course of this work it became apparent that certain of the southern provinces of Upper Egypt offered unusually favourable opportunities for the study of the epidemic relations of pneumonic plague.

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A "NEW" SALMONELLA FROM A CASE OF ENTERIC FEVER.

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(With 4 figures.)

THE list of bacteria recorded as causative agents in enteric fever and "food-poisoning" increases steadily, and the proved pathogens already number eleven types, of which three have been found in both continued fever and food-poisoning, three in continued fever alone, and five in food-poisoning alone. This list will undoubtedly be added to when cultural methods for the diagnosis of these conditions are more widely employed, especially in the tropics.

Unfortunately bacteriologists are not agreed on the delimitation of the salmonella group which includes nine of the eleven types (*B. typhosus* and *B. paratyphosus* A excluded); some workers restrict the genus (?) *Salmonella* to bacilli having certain cultural characters in common, but more or less distinctive serological reactions, others include, on serological grounds, organisms such as *B. gallinarum*, *B. glässer*, and *B. voldagsen* which do not present the cultural characters of the "true" salmonellas, and American writers have included *Morgan's bacillus* which has neither cultural nor serological connection with the group and could scarcely be admitted even on the ground of pathogenicity.

For the purposes of this paper the salmonellas may be regarded as presenting the following common characters: Gram-negative non-sporing bacilli, usually actively motile, which do not ferment lactose, saccharose or salicin, do not liquefy gelatin, and never give the indol reaction. In litmus-milk they cause a transient acidity followed after about 48 hours by alkalinity. They ferment glucose, mannitol, and maltose with production of acid and gas. These general reactions cover salmonellas in the restricted sense (Topley, Weir and Wilson, 1921).

In the case to be described a bacillus presenting definite affinities with the salmonella group was isolated from the circulating blood of a patient suffering from typical enteric fever. The apparent absence of the various bacilli commonly causing this disease, and the fact that the patient's serum did not react with any of them, but only with the homologous bacillus, seem to justify the assumption that this bacillus played the role of pathogen.

The case. On October 14th, 1922, an Indian seaman, aged 28, was admitted to the Royal Albert Dock Hospital under the care of Dr G. C. Low. The patient presented all the common clinical features of enteric fever, and during his first

week in hospital suffered two attacks of intestinal haemorrhage. After this he made an uninterrupted recovery; the illness running the usual course of a mild typhoid. Unfortunately no history prior to his admission to hospital is obtainable, but as he was admitted directly from his ship in the early stage of the disease, it seems probable, taking into account the duration of the voyage, that the infection was contracted in Bombay, if the man were not already a carrier.

Widal tests made during the first and third weeks in hospital were negative with different strains of *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B, *B. paratyphosus* C, *B. aertrycke* (Mutton and Newport types) and *B. enteritidis* Gärtner, but *B. suispestifer* (Hog Cholera Ten Broeck, No. 16) was agglutinated in titres up to 1/80.

The bacillus. A blood-culture was successfully made on the day of admission and a bacillus (hereinafter referred to as the *Alphonso* strain) having the following characters was obtained in pure culture. Morphologically like *B. typhosus* and the salmonellas. Actively motile, Gram-negative, gelatin not liquefied, and the indol reaction not given. Lactose, salicin and dulcitol were not fermented. Litmus-milk was rendered acid, changing to an alkaline reaction after 48 hours. Glucose, mannitol and maltose were fermented with acidity and gas evolution. It therefore presents the general characters of the salmonella group, and in its failure to ferment dulcitol it resembles *B. suispestifer*.

Agglutination tests. The patient's serum taken five weeks after admission to hospital agglutinated the bacillus up to a titre of 1/500, but agglutination at all titres was incomplete, a portion of the suspension remaining unagglutinated. Agglutination also occurred with certain stock sera for the group: with *B. enteritidis* Gärtner serum to 1 per cent. of full titre, *B. paratyphosus* B serum 16 per cent., *B. paratyphosus* C serum 10 per cent., *B. aertrycke* mutton serum 10 per cent., *B. glässer* serum 10 per cent. It was not agglutinated by sera for *B. typhosus*, *B. paratyphosus* A, or the *Newport bacillus*, nor did it absorb the specific agglutinin from any of the stock sera mentioned.

Pathogenicity to albino rats. Tests were made when the strain had been cultivated under artificial conditions for three months. Oral feeding was apparently without effect, but 0.25 c.c. of a very young broth culture introduced into the peritoneum caused death in four days. Post-mortem the only macroscopic changes noticed in the animals were petechiae in the lungs and some enlargement of the spleen; the peritoneum appeared normal. The bacillus was recovered in pure culture from the spleen, liver, and heart and was not found to have been modified in any respect by its passage through the animal.

Tests with high-titre sera. An *Alphonso* agglutinating serum, (rabbit), having an end-titre for the homologous bacillus of 1/20,000, was prepared, and tested with the following 11 types obtained from the National Collection of Type Cultures. The numbers refer to the catalogue of the National Collection.

404 A "New" *Salmonella* from a Case of Enteric Fever

B. paratyphosus A (Schottmüller). No. 13.

B. paratyphosus B (Tidy). No. 14.

B. paratyphosus C (East Africa) *Salmonella* type Hirschfeld, No. 777.

Hog Cholera bacillus (Ten Broeck No. 19) *Salmonella* type Hirschfeld. No. 357.

B. enteritidis Gärtner (Danyz). No. 205.

B. aertrycke, *Salmonella* type Mutton. No. 115.

Salmonella type Newport. No. 129.

Salmonella type Binns. No. 73.

Salmonella type Stanley. No. 92.

Salmonella type Reading. No. 72.

Salmonella type "G." No. 91.

These strains were all agglutinated in varying degree by the *Alphonso* serum; see Fig. 1.

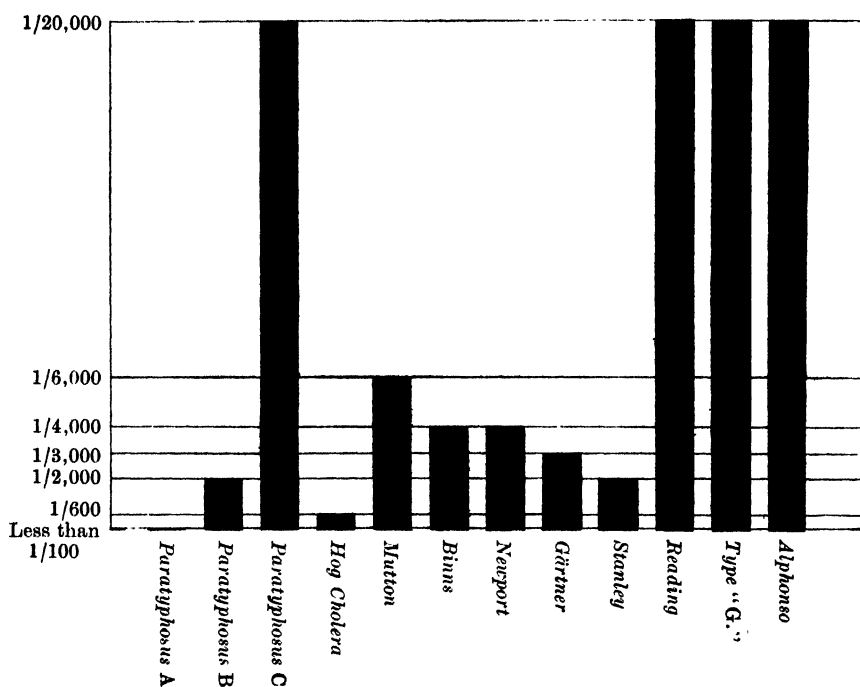


Fig. 1. Agglutination of twelve salmonellas by *Alphonso* serum. End-titres of agglutination: *B. paratyphosus* A less than 1/100. *B. paratyphosus* B 1/2000. *B. paratyphosus* C 1/20,000. *Hog Cholera bacillus* 1/600. *Mutton* type 1/6000. *Binns* type 1/4000. *Newport* type 1/4000. *B. enteritidis* Gärtner 1/3000. *Stanley* type 1/2000. *Reading* type 1/20,000. *Type "G."* 1/20,000. *Alphonso* 1/20,000.

The individual agglutinability of all these strains, except that of the *Hog Cholera bacillus*, was good. Agglutination in every case was incomplete, especially in the higher titres.

Absorption of *Alphonso* serum by the various types separately affected no reduction of end-point for the homologous bacillus except in the cases of *Type "G.," Reading*, and *Newport* (Fig. 2). *Type "G."* removed all agglutinin at 1/50, *Reading* reduced the end-titre for *Alphonso* to 1/3000, but did not

completely remove all group-agglutinin for any of the types except itself and *gärtner*.

Although absorption by *Paratyphosus* B, *Paratyphosus* C, *Stanley*, *Binns*, and *Mutton* caused no apparent reduction in end-titre for *Alphonso*, the quality and the velocity of agglutination were affected; as compared with a control (unabsorbed) serum agglutination was much slower and more incomplete, the flocculi being very minute and fewer in the higher titres especially after absorption by *Paratyphosus* C. This phenomenon is well known and was noticed by Castellani in 1902, in the case of a *B. typhosus* serum of titre 1/10,000 which

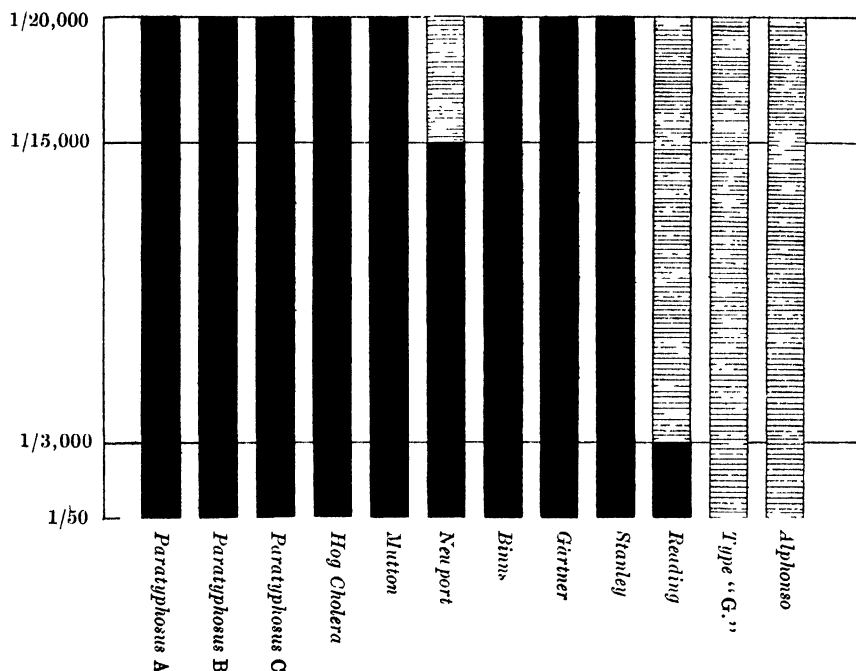


Fig. 2. Effect of absorption of *Alphonso* serum by twelve salmonellas separately, on end-titre for homologous bacillus. Absorption was carried out in a titre of 1/50 and was complete in the cases of Type "G." and *Alphonso*.

also agglutinated *B. coli* up to 1/800. Absorption by *B. coli* removed all of the *coli* agglutinin without lowering the end-titre for *B. typhosus*, but the clumps of *B. typhosus* were small as compared with the clumps formed in the unabsorbed serum.

The five types mentioned therefore appear capable of removing some of the agglutinins effective for *Alphonso* without lowering the end-point. These agglutinins evidently represent the elements by which the organisms are themselves agglutinated and seem to correspond to the more remote type of group-agglutinin or *co-agglutinin*. That this *co-agglutinin* is not a single element but a mixture of agglutinins corresponding to the apparently complex antigenic

406 A "New" *Salmonella* from a Case of Enteric Fever

mosaic of the *Alphonso* bacillus is suggested by the fact that each of the five types can remove completely only its own co-agglutinin, but not completely those for other types. This is illustrated in Fig. 3 which shows the effect of absorption of *Alphonso* serum by *Paratyphosus* C on agglutination for the group.

It will be seen that the results of the absorption tests arrange themselves into three groups: (1) complete absorption of all agglutinins by *Alphonso* and Type "G."; (2) reduction of end-titre for the homologous bacillus by *Reading*

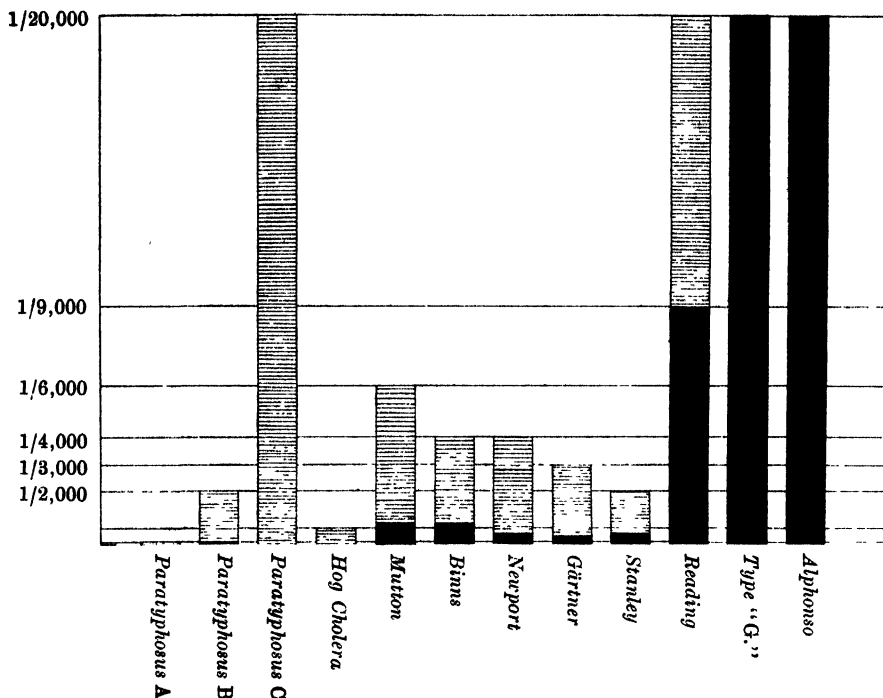


Fig. 3. *Alphonso* serum absorbed by *B. paratyphosus* C at 1/50. Effect on end-titres of agglutination for the twelve salmonellas. The light shading indicates the reduction in titre, the dark shading the titre of agglutination.

Alphonso and Type "G." no reduction in end-titre. *Reading* reduced from 1/20,000 to 1/9,000. *Stanley* from 1/20,000 to 1/400. *Gärtner* from 1/30,000 to 1/300. *Newport* from 1/40,000 to 1/400. *Binns* from 1/40,000 to 1/800. *Mutton* from 1/60,000 to 1/800. *Hirschfeld* type (*Paratyphosus* C and *Hog Cholera*) complete absorption. *B. paratyphosus* B from 1/20,000 to 1/100.

and *Newport*; (3) alteration in the quality of agglutination without reduction of end-titre by each of the others.

The *Alphonso* serum therefore may be said to contain a "specific agglutinin" common to *Alphonso* and Type "G." an "intimate group-agglutinin" removable by *Reading* and to a slight extent by *Newport*, and a more remote type of group-agglutinin or co-agglutinin affecting the other types. It has already been suggested that the co-agglutinin is made up of several different elements

corresponding to the different antigenic types, and these elements appear to run parallel so that absorption by a single type, removing chiefly its own co-agglutinin, does not lower the end-titre, but if two types be employed together the effect is increased; that is the degree of agglutination in the higher titres is still further lessened, and agglutination at full-titre is very feeble or doubtful. When combined absorption by four of these types (*Paratyphosus* B, *Paratyphosus* C, *Mutton* and *Binns*) was attempted there was a definite lowering of the end-point; agglutination at 1/15,000 being a mere trace and even at 1/10,000, the flocculation was not more than half as marked as at 1/20,000 of the control (unabsorbed) serum. This lowering of the end-point by combined absorption cannot be attributed to the greater mass of bacteria employed, as absorption by a similar mass of any of the types separately was without such effect.

Combined absorption at 1/10 by *Paratyphosus* B, *Paratyphosus* C, *Gärtner*, *Mutton*, *Newport*, *Stanley*, *Binns*, and *Reading* removed all agglutinin except that for *Alphonso* and *Type* "G." and the end-titre for these two was reduced to 1/400. This probably represents the full titre of *specific agglutinin*. *Reading* and *Newport* together did not lower the titre below the *Reading* figure 1/3000, and the further reduction to 1/400 must have been brought about by the other types.

A *Type* "G." serum having an end-titre of 1/30,000 was prepared. This serum was as catholic in its affinities as the *Alphonso* serum and gave, even with the homologous bacillus, a similar incomplete type of agglutination. Fig. 4 shows the agglutination of the group by this serum, and it will be seen that, in the types most affected, its action is very similar to that of the *Alphonso* serum.

At a titre of 1/100 *Type* "G." serum was completely absorbed by *Alphonso*.

Salmonella Type "G." was isolated at the Lister Institute (1917) from a mesenteric gland of a monkey which died in the course of a dietetic experiment.

Some time before the examination of the *Alphonso* strain was completed, a culture and some of the serum were sent to Sir Frederick Andrewes who tested them with *mono-specific* and *ultra-specific* sera and *specific* (*sensu stricto*) types of the common salmonellas (but not *Type* "G."). Sir Frederick Andrewes has very kindly given me permission to state that he has not been able to identify *Alphonso* strain with any of these. Unfortunately, repeated attempts to obtain the *specific* type of the bacillus were unsuccessful.

The results of the serum tests described may be taken as definitely establishing the identity of the *Alphonso* bacillus as a strain of *Type* "G." It should however be mentioned that when cross-absorptions were attempted at a serum dilution of 1/10, it was found in the case of the *Alphonso* serum that although absorption by *Type* "G." removed all agglutinin for this strain, a trace active for *Alphonso* remained even after repeated re-absorption by *Type* "G."; the homologous bacillus itself sometimes failed to remove this trace. Similarly absorption of *Type* "G." serum at this titre by *Alphonso* left a trace of agglutinin

408 A "New" *Salmonella* from a Case of Enteric Fever

which however was active for both strains. This should not be regarded as indicating any racial or sub-strain difference between the organisms but rather as the natural consequence of attempts to absorb from too great a concentration of a high-titre serum: Eisenberg (1903) showed that absorption of a high-titre serum by a single dose, no matter how great, of the homologous bacillus would not remove *all* agglutinin, and that repeated absorption may sometimes leave a trace of *residual* agglutinin.

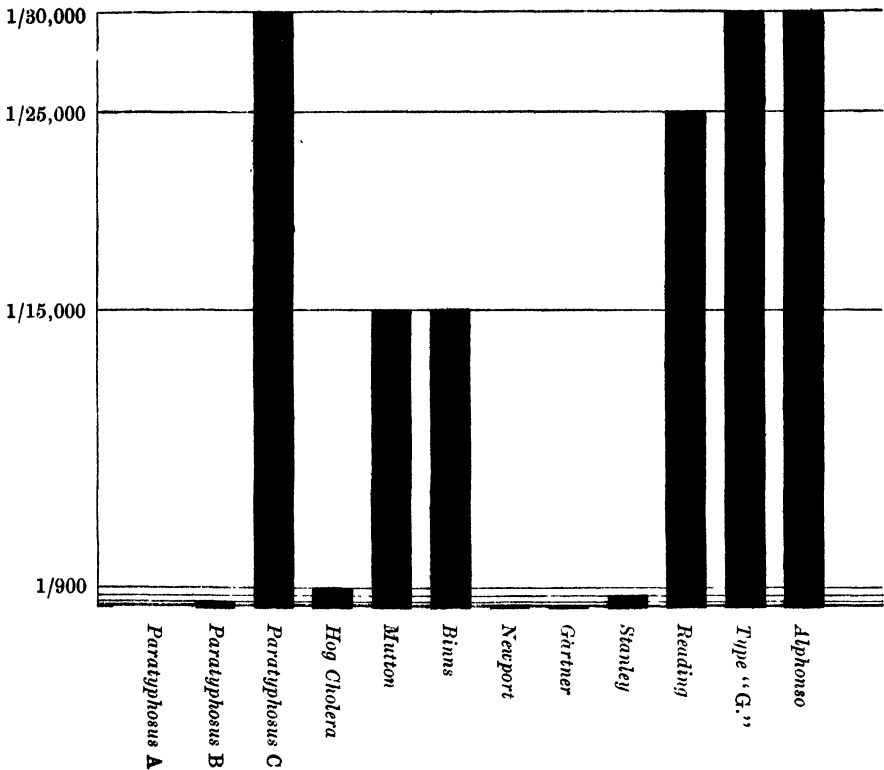


Fig. 4. Agglutination of twelve salmonellas by Type "G." serum. End-titres of agglutination: Type "G." 1/30,000. Alphonso 1/30,000. Type Reading 1/25,000. Type Stanley 1/600. *B. enteritidis* Gärtner 1/100. Type Newport 1/150. Type Binns 1/15,000. Type Mutton 1/15,000. Hog Cholera bacillus 1/900. *B. paratyphosus* C 1/30,000. *B. paratyphosus* B 1/300. *B. paratyphosus* A negative. Agglutination, as in the case of the Alphonso serum, was *incomplete* with all strains.

Since its isolation the *Alphonso* strain has always been very *unspecific* and it is desirable that the results of the serum tests should receive confirmation from the examination of a *specific* type. Up to the present all attempts to isolate the specific type have failed, and as the strain in my hands has recently undergone further antigenic degradation, evidenced by a marked reduction in agglutinability and absorbing power, the probability of obtaining such a type seems more remote.

Examined by new biochemical tests devised by Major H. C. Brown, C.I.E. and myself for the differentiation of the salmonellas, *Alphonso* shows some relationship with *Paratyphosus* C and *Reading*, and it is indistinguishable from *Type* "G." and the Hog Cholera bacillus; these three also give similar reactions with the common fermentation tests. In this connection it is worth repeating that the Hog Cholera bacillus was the only heterologous organism agglutinated by the patient's serum, and although the tests with the high-titre serum did not establish any connection between it and *Alphonso*, it should be mentioned that the only Hog Cholera strain available has undergone great retrogressive change and for serological work is of little value. It is not possible therefore to say what relationship, if any, may exist between *Alphonso-Type* "G." and the Hog Cholera bacillus.

In evaluating the tests a few points in the technique must be stated. In the preparation of the high-titre sera formolised or carbolised suspensions, *sterilised in the cold*, were used. For absorption 6 in. agar plates were sown from agar cultures. The preliminary absorbing dose used was a 48 hours' growth from three or four of these plates to 3.0 c.c. of a 1/50 dilution of serum; the growth being either washed off with the already diluted serum or scraped off and added to it. The absorbing mixture was kept in the cold chamber for two days before centrifuging. In titres below 1/50 re-absorption was done. For all agglutination tests stock suspensions of 20 hour peptone water cultures fixed with 0.1 per cent. formol and diluted to a density about 100 per cent. greater than that of the "Standard" suspensions of the paratyphoids were used, and the tests were incubated at 55° C. for three hours. The denser suspensions were made necessary by the low specificity of the *Alphonso* and *Type* "G." strains.

I wish to express my indebtedness to Sir Frederick Andrewes for kindly allowing me to mention the results of his *specific* tests, to Dr G. C. Low for permission to refer to the clinical case, and to Dr P. H. Manson-Bahr from whom I received the culture.

CONCLUSIONS.

- (1) The *Alphonso* bacillus is a *Salmonella* and apparently a strain of *Type* "G."
- (2) It was the cause of a typical enteric fever.

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ON THE PRODUCING OF MILK HAVING A LOW BACTERIAL CONTENT.

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ABSTRACT¹.

ABOUT a year ago the specific opportunity which our laboratories had been seeking presented itself. We were brought into touch with a farm on the outskirts of Vancouver engaged in the production of milk and the distributing of the same in the city. The proprietors of the farm, with at that time a herd of twenty-five grade cows, were endeavouring to supply a high grade milk, a milk having a low bacterial content, and secured from cattle giving a negative reaction to the tuberculin test. We approached the proprietors, Mr and Mrs Alexander Hill, and found that they were desirous of co-operating with us. At the same time their milk was obtaining a premium over the usual price obtained for market milk sold in the city. We made it clear that we were anxious to secure data which would be of use to milk producing farmers generally with respect to the possibility of producing a high quality milk; and they—Mr and Mrs Hill—assured us that they desired to have advice and help if the same could be forthcoming. Finally we made the following arrangement: (a) we were to be permitted to obtain samples of milk at any time deemed most suitable to ourselves, (b) these samples were to be examined for bacterial flora to the end that we might secure the data we needed, (c) no report on the examinations was to be sent from our laboratories until it became possible for us to publish the data as a whole, (d) the proprietors of the dairy were to be given any information that was available from the laboratory data, but, under no circumstances, was the same to be used for publicity purposes, (e) we were to check up any of the procedures in vogue on the farm and dairy as far as proved to be possible without unduly interfering with the progress of our main project. The arrangement was agreed to mutually, and it is a matter of gratification to us that throughout the entire investigation the conditions detailed above were subscribed to loyally.

We conducted preliminary work in February and March, 1923, and the investigation proper commenced on April 10th, 1923, and continued until July 24th, 1923².

¹ Sadler, Wilfrid, Kelly, C. D. and Martin, G. R. (1923). On the Producing of Milk having a Low Bacterial Content. *Scientific Agriculture*, Ottawa (in Press).

² During the past summer, the senior worker was absent from the University, and the laboratory work was done by Mr C. D. Kelly and Mr G. R. Martin. Mr Martin is conducting a detailed study of certain of the organisms isolated and retained during the work. W. S.

The farm premises would be held to be well below the average of what are usually considered to be suitable holdings for the production of milk. In the paper (*loc. cit.*) a description of the premises, of the methods and procedures followed by the proprietors and of the system adopted by them in the cleansing and sterilization of utensils, are given in detail. A milking machine was used throughout. A small quantity of the first milk was drawn into a separate pail and in all cases this was used for the feeding of certain livestock. The milking as such was done with the machine. Stripping was done by hand and the strippings were mixed with the milk supply.

Throughout the period during which the work was carried on, 90 samples of milk were examined, three samples being taken on each of the 30 days when determinations were made. At each sampling, two pint bottles of milk, intact, as bottled by the dairy, were taken by us on the premises. One bottle was of evening's milk—designated I in Table, and one was of morning's milk—designated II in Table. The bottles were chosen at random, and usually were obtained by us at 8.30 a.m. In addition, a sample of morning's milk after cooling and prior to bottling and representative of the entire supply, was taken—designated III in Table. The media employed for the plate counts was the Bacto-purple-lactose-agar (dehydrated) of the Digestive Ferments Company. For detecting lactose-fermenting (gas-producing) types we employed MacConkey's neutral-red-bile-salt-broth. All determinations were done in triplicate. Triplicate plates were incubated at 37° C. and at 22° C. respectively. The former were counted after 48 hours' incubation and the latter after 5 days' incubation. For the fermentation tests, triplicate tubes containing 20 c.c. of milk in each case were submitted to the fermentation test at 37° C. and at 22° C. respectively. For the reductase test triplicate tubes containing 20 c.c. of milk and 0.5 c.c. methylene-blue in each case were submitted to the methylene-blue-reductase-test at a temperature of 37° C.

DATA OBTAINED.

Of the 90 samples examined, 7 samples gave a count of 8150, 7800, 5050, 5260, 5800, 5800, and 5400 colonies per c.c. respectively; 83 of the 90 samples gave a count of less than 5000 colonies per c.c. The counts are recorded as for the plates incubated for 48 hours at 37° C. The appended Table summarizes the averages of the counts for each set of samples for the period April 11th to June 6th and June 6th to July 24th respectively. When average counts are considered, it is seen that the highest average for any one period is 3680 colonies per c.c. and that throughout the work in all instances for any period considered, the count was below 5000 colonies per c.c. We failed to find organisms of the lactose-fermenting (gas-producing) types in 1/10 c.c. of any sample of milk examined. In only 5 of the 90 samples did we find gas-producing types in 1 c.c. of the milk. The results from the fermentation tests and from the reductase test agreed in the main with the findings from the plate counts. Our results as a whole, considering the data available from all our determina-

412 *Producing Milk having a Low Bacterial Content*

Table.

				PLATE COUNTS. No. of colonies per c.c. Average for no. of samples specified in column 3		PRE- SUMPTIVE TEST. Gas-pro- ducing types in 1/10 c.c. milk	RE- DUCTASE TEST Hours required to re- duce meth blue 37° C.	FERMENTATION TEST. No. of hours.			
Samples	Dates of sampling 1923	No of samples examined	Treatment of utensils	Incubated at 37° C.	Incubated at 22° C			Before milk clotted		Before peptonization observed	
								37° C.	22° C.	37° C.	22° C.
I. Bottle of evening's milk	Apr. 11 to June 6	14	Utensils steri- lized once a day	2000	2130	0	23	43	86	27	59
	June 6 to July 24	16	Utensils steri- lized twice a day	3040	3460	0	24	35	101	23	61
II. Bottle of morning's milk	Apr. 11 to June 6	14	Utensils steri- lized once a day	2920	3650	0	24	43	101	24	63
	June 6 to July 24	16	Utensils steri- lized twice a day	2740	2660	0	23	38	105	22	62
III. Sample of morning's milk	Apr. 11 to June 6	14	Utensils steri- lized once a day	3120	3680	0	23	42	104	25	63
	June 6 to July 24	16	Utensils steri- lized twice a day	2470	2660	0	23	37	101	22	61

tions, indicate that the milk produced on the farm under consideration is of excellent quality bacteriologically. It is our conviction, based on the work recorded, that the results are indicative of the paramount importance which is to be attached to the quality of the personnel, and to the effective cleansing and sterilization of utensils as factors influencing the bacterial quality of milk. The results of our work appear to harmonize with those recorded by North, Delépine, Ayers, Cook and Clemmer, Stenhouse Williams and others.

It remains to be said, that the milk produced and distributed by the farm dairy under consideration commands an enhanced price in the City of Vancouver.

(*MS. received for publication, 24. 1. 1924.—Ed.*)

ON THE CONCENTRATION OF SERUM BY MEANS OF SODIUM SULPHATE.

BY A. T. MACCONKEY.

Lister Institute (Serum Department).

THE method most commonly employed for the separation of antitoxic globulins is that by means of Ammonium Sulphate, and this was the process used in these laboratories for several years until, at the end of 1917, the supply of the necessary salt was cut off. We were then thrown back on Sodium Sulphate, a salt we had used before the War but which had not given us satisfactory results. A few fresh trials, however, showed us the faults of our previous technique and proved that Na_2SO_4 could satisfactorily replace Am_2SO_4 and so, since February 1918, we have used only the former salt. Changes in detail have been made from time to time and we have now established a routine method which has for some time given us satisfactory results.

The object of this communication is to describe the process as it may be of use to others who are interested in the same subject. To those who are conversant with the literature of the concentration of serum it will be obvious that the method is based on the work of S. N. Pinkus (1901-1902) and on that of R. Gibson (1905).

Before describing the routine method it may be as well to mention that, as is the case with ammonium sulphate, "fractional" precipitation by Na_2SO_4 allows of the antitoxic globulins being separated out and a considerable, say eight to ten times, concentration realised. But it must be borne in mind that the precipitation by Na_2SO_4 must be carried out at a temperature above 33°C . because below this temperature the concentration of the salt necessary for the precipitation cannot be obtained.

At $33\text{--}40^\circ\text{C}$. the addition per 100 c.c. of plasma or serum of 10-11 gms. Na_2SO_4 will throw down the euglobulin and the further addition per 100 c.c. of filtrate of 8.5-7.5 gms. of sulphate will separate the pseudoglobulin, the albumen remaining in the filtrate. The precipitation limits are not sharp and some of the antitoxin is carried down with the euglobulin and a little may pass on with the albumen. If, then, we take into account only the fraction defined by the limits 11 and 18.5 gms. of Na_2SO_4 we can obtain high concentration, but a fairly large loss of antitoxin must be expected. If it is considered worth while much of this loss can be recovered by appropriate treatment of the precipitate, and in our routine process we make this our aim, but when all has been done it will be found that if a minimum loss of antitoxin is desired one must be content with a concentration of four to five times the original volume. We,

414 *Concentration of Serum by means of Sodium Sulphate*

here, are satisfied with this increase, and it is only in special cases that we work for the higher degree.

As an example of the possibilities of this fractional process, I mention that we have obtained from an antitetanic serum containing 250 units per c.c. a solution of antitoxic globulins of a titre of 2200 units per c.c.

The usual routine proceeds as follows:

Plasma¹ or serum is warmed in a water bath to 33–37° C. and then anhydrous sodium sulphate is added and dissolved until the density reaches 1175, which represents about 18·5 gms. of sulphate per 100 c.c. of plasma or serum. The mixture is then filtered through chain cloth at a temperature of 33–37° C. and allowed to drain thoroughly. In the precipitate will be found euglobulin, pseudoglobulin and sulphate; in the filtrate, albumen and sulphate.

The precipitate is re-dissolved in water at 33–37° C. in the water bath, made up to the original volume of the plasma and the precipitation and filtration repeated.

The albumen filtrates may be discarded. The precipitate of euglobulin and pseudoglobulin is now dissolved in cold water and made up to twice the original volume of plasma. When completely dissolved common salt (table salt²) is added to saturation. The density should then be 1200–1205. The mixture is filtered through chain cloth. The precipitate is again dissolved up in water, made up to the original volume of plasma and the process of saturation with salt, and filtration repeated.

The residue on the filter cloths (euglobulin) may be discarded, or digested and used for media making, or used as a fertilizer. The two portions of filtrate (solutions in brine of pseudoglobulin) are mixed together and the globulin is precipitated by the addition of 0·3 per cent. of glacial acetic acid.

The precipitate is separated by filtration through chain cloth and allowed to drain thoroughly.

The cloths with the precipitate are opened out flat and placed together face to face (precipitate inside) in pairs between towels to remove the excess moisture—the towels being changed as often as may be necessary.

When fairly dry the cloths, still in pairs, are placed between dry towels, and these between boards in a press, there being alternately a board and a pair of cloths.

Slight pressure only is put on at first and then gradually increased as the

¹ The plasma or serum may be diluted with water if preferred. The ordinary reaction of plasma or serum is a quite suitable one.

² The table salt used is supplied by Messrs Bumsted and Co., who have kindly sent us the following recent analysis:

Sodium chloride	99·86 %
Sodium sulphate	0·11 %
Calcium sulphate	free
MgCl ₂	„
Sodium carbonate	0·01 %
Sodium bicarbonate	0·02 %
Insoluble	none

precipitate becomes drier. When properly dried the precipitate is like crumbling cheese—neither pasty nor hard and gluey.

Great care must be taken not to overpress as this spoils the result.

The dried precipitate is removed from the cloths, weighed out in lots of 800–1000 gms. and placed in the centre of moistened circular (24 ins. diameter) sheets of vegetable dialysing parchment. Powdered washing soda is then sprinkled over it in the proportion of 3 per cent. of the weight of the precipitate. The parchment paper is folded up into the form of a bag and suspended in a dialysing tank through which water gently flows.

When free from excess of salts—which usually takes place in about 48 hours and which may be ascertained by the dipping refractometer¹—the pseudoglobulin solution is removed from the dialysing bags and measured. Then 0·7 per cent. of a mixture of equal parts of cresylic acid and ether (Banzhaf, 1921) is added and the solution is stored in sterile 4 litre corked bottles in the cold room until required.

EXAMPLES OF RESULTS.

Batch 1.

Diphtheria antitoxin.

550 litres of plasma were mixed together and treated as above at the rate of about 100 litres per week. The results of the several weeks' work were mixed together and tested:

Original 550 litres at 250 a.u. per c.c. = 137,500,000 a.u.

Concentrated 96·5 litres at 1400 per c.c. = 135,100,000 a.u.

Batch 2.

Original 500 litres at 350 a.u. per c.c. = 175,000,000 a.u.

Concentrated 168·5 litres at 1000 per c.c. = 176,925,000 a.u.

Tetanus antitoxin.

Original 110 litres at 200 a.u. per c.c. = 22,000,000 a.u.

Concentrated 23·3 litres at 1000 a.u. per c.c. = 23,300,000 a.u.

Antidysentery serum.

Original 200 litres neutralising 500 M.L.D.'s (rabbit) per c.c.

= 100,000,000 M.L.D.'s.

Concentrated 50 litres neutralising 2000 M.L.D.'s (rabbit) per c.c.

= 100,000,000 M.L.D.'s.

Antiscorpion serum and antiplague serum can be concentrated in the same way.

The question may be raised as to how it comes about that in diphtheria antitoxin, batch 2, mentioned above, the solution after concentration contains a larger total number of units than were apparently present originally.

¹ A portion of the liquid in the dialysing bags is diluted with an equal quantity of distilled water, boiled and filtered. The salt content of the filtrate is estimated with the refractometer and compared with a filtrate obtained by treating normal serum in the same manner.

416 *Concentration of Serum by means of Sodium Sulphate*

It stands to reason that no process of this sort can be carried out without some loss actually occurring. The figures given above are not meant to show that no loss has taken place. The sera are not tested closer than 50 units. Thus the titre of the original plasma in batch 2 was 250 units but not 300, while that of the concentrated solution was tested to be 1050 units but not 1100 units per c.c. The possible differences in the actual exact titres would easily account for the apparent excess recovered, but the maximum possible loss could not have been more than 11.5 per cent.

The degree of concentration obtained depends upon the pressing and drying of the pseudoglobulin precipitate and the greater the concentration the higher will be the percentage of protein in the final product.

We have found that sodium sulphate has the following advantages over ammonium sulphate:

1. It is non-toxic and therefore it is not so necessary to remove the last traces of it¹.
2. It is not such a good food for micro-organisms and so there is not the same liability to contamination and it is not necessary to put any preservative in the dialysing bags.
3. The rate of dialysis is quicker (*cf.* Banzhaf, 1908-9).
4. With equal concentration of protein the final product is not so viscid.
5. The cost is less.
6. The sodium salt does not attack metal so strongly.

The description may give the impression that the process is complicated and laborious but we do not find it so in practice. We have adopted it only after a lengthy trial and comparison with other methods. During the war, when we were using ammonium sulphate in various ways, we came to the conclusion that the old original Gibson process was the one which gave the best and most reliable final product, and now it will be seen that after wandering in many devious by-paths we have found our way back to, and are content to travel on, the old road, but substituting sodium for ammonium sulphate.

I am much indebted to Mr Albert Riggs, head laboratory assistant, for his enthusiastic co-operation in the work and for the very careful observations he made during experiments, observations which resulted in valuable practical improvements in the technique.

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¹ 1 c.c. of a 7 % solution of sodium sulphate injected intraperitoneally into a mouse did not cause death but 1 c.c. of a 10 % solution killed in 1½ hours. (*Cf.* also F. Greenwald, 1918, *Journ. Pharmac. and Experiment. Therapeutics*, vol. xi. p. 283.)

TYPHOID CARRIERS IN ABERDEENSHIRE.

By JAMES P. WATT, M.A., M.D., D.P.H.,

Medical Officer of Health for Aberdeenshire.

IN the course of investigations into outbreaks of typhoid in the county for which I act as Medical Officer, I have discovered a number of typhoid carriers whose histories seem worth placing on record as showing the extent to which the endemicity of the disease in rural areas is maintained by carriers. Of twenty-two found, four were identified in 1908, two in 1910, three in 1911, one in 1916, one in 1917, five in 1918, and six in 1919. Five were men and seventeen were women, giving a proportion of 77·3 per cent. of female carriers. Of the chronic typhoid carriers found by the German stations up to the close of 1907, 82 per cent. were women. All of my carriers were intestinal, one being a urinary carrier as well. As in every case attention was directed to the carriers owing to a case or cases of actual typhoid fever occurring; this experience indicates that, as is generally believed, faecal carriers are altogether in excess of urinary carriers, or that urinary carriers may not have the special malignancy commonly attributed to them.

A rural area affords special facilities for the discovery of typhoid carriers owing to the fact that outbreaks tend to recur at the place where the carrier is and that the movements of carriers and patients and their contact with others can be readily determined, while the number of persons among whom the carrier has to be searched for is comparatively limited. "Carriers," says Gay⁽¹⁾, "usually become recognised by careful epidemiological surveys in connection with repeated cases of typhoid fever in a given locality, in association with some particular individual over a period of time, or in connection with some epidemic due to food, in the preparation of which he, or more frequently she, has taken part. In such surveys it is usually possible by a process of elimination to identify the carrier or carriers in a given group of individuals. The method by which this elimination is carried on will depend to a large extent on the individual skill, tact, and experience of the epidemiologist." The chief difficulty lies in obtaining the samples of stools and urine necessary for identification of the typhoid state, and in the intermittency of the discharge of the bacilli often rendering several samples necessary. As there have been no compulsory powers, and it is not clear if there are yet any, to enable one to get these samples where refused, they could be obtained only by favour.

In view of the tragic position of the chronic typhoid carrier, it is a matter of profound regret that all the attempted lines of treatment have met with so little success. Ledingham and Arkwright⁽²⁾ conclude their review of the various

measures tried with the remark, "We have to deplore the fact that so far the attempts to cure intestinal carriers have not yielded results affording convincing evidence of their success. In the case of carriers who are in an early stage of this condition, there may be some hope of effecting a permanent cure by one or other of the methods already tried and quoted above, but in long standing chronic cases the prospect of success of this kind would seem to be extremely remote." According to Nichols(3), removal of the focus of the disease by surgical operation has been successful in over 50 per cent. of typhoid carriers. For carriers who are in advanced years, and many of them are so, operation would not be advisable. It cannot therefore be too earnestly hoped that a treatment will ere long be found which will obviate the need for surgical measures.

I propose in this paper to give an account of the carriers in the order in which they were met with, and to make some observations on the present position of the law in Scotland as regards typhoid carriers.

TYPHOID CARRIERS.

Year, 1908.

Case 1. Mrs A., wife of tenant of Farm B.

The following outbreaks of typhoid fever had occurred at this farm:

1892.	Nov.	2nd.	Daughter, aet. 13 months.
		2nd.	Husband, aet. 28 years.
		2nd.	Daughter, aet. 3 years.
		14th.	Farm servant, aet. 35 years.
1897.	Oct.	3rd.	Farm servant. Died.
1906.	Nov.	6th.	Daughter, aet. 9 months.
1908.	Aug.	7th.	Farm servant, aet. 27 years.
		7th.	Farm servant, aet. 15 years.
		8th.	Domestic servant, aet. 17 years.
		16th.	Daughter.

Between 1892 and 1906, there had been cases of illness in the family which the medical attendant, in the light of subsequent events, was inclined to look back upon with suspicion as having been probably typhoid, although he had not at the time diagnosed them as such. Only two children out of a large family would seem to have escaped an attack.

In 1908, a sister-in-law of Mrs A., who had been staying with her family of four daughters for summer holidays in a village near, developed typhoid immediately after her return home, as did also two of the daughters. They had received part of their milk supply from Farm B. I found, however, subsequently that the woman in whose house they were lodging, and from whom they got part of their milk, was also a typhoid carrier so that infection might have come from either source.

The examination of samples of stools and urine which I obtained in connection with the outbreak in 1908 showed Mrs A. to be a faecal carrier. She had had an attack of typhoid in 1890, shortly before her marriage. Her

husband entered Farm B. in 1892, and no outbreak of typhoid was known to have occurred there before that date.

In 1911, another case of typhoid occurred at B., a man-servant, age 19, and in 1914, a young man of 20 living in a neighbouring village who had been working for a time at B. went home from there ill, and the illness proved to be typhoid. No cases have since occurred at B. or been connected with the farm.

I may mention that a new water supply had been introduced prior to 1906.

Case 2.

The second case identified in 1908 was a woman A. P., age 63, who was acting as housekeeper to a farmer, Mr S. Mr S. was about to be married when he developed typhoid, was removed to hospital on August 31st, and died on September 16th, 1908. A. P. was known to have been working in houses in the county where previous outbreaks had occurred, and suspecting her to be a carrier, I persuaded her to go to hospital where samples of her stools and urine were taken and sent to the laboratory for examination. The stools proved to be positive.

A. P. had passed through a severe attack of typhoid in 1900, while a patient in Banff Asylum. This asylum, as related by Ledingham and Arkwright(2), had, between the years 1893 and 1907, been visited by eleven outbreaks of typhoid with a total of 31 cases. In 1907, a systematic search for carriers was undertaken by Drs A. and J. C. G. Ledingham, and three carriers were discovered among the female patients. These were isolated, after which the outbreaks ceased¹.

Shortly after her attack of typhoid, in the end of 1900, A. P. was discharged from the Asylum and went to reside for a time at a farm in the vicinity, in the Parish of Kirkmichael. Here occurred the first outbreak with which she was connected. I ascertained through an enquiry kindly made for me by Dr Ledingham, Medical Officer of Health for the county, that during her stay there she assisted occasionally in the work of the kitchen and took the kitchen servant's place when the latter, who was the first known case, became ill. This outbreak produced 13 cases. The following account of it is taken from the *Report* for 1901 of Dr Cameron, Medical Officer of Health for the county:

The most serious outbreak occurred in the Kirkmichael, Tomintoul and Glenlivet Districts. A servant girl was taken ill at a farm in Kirkmichael in April, and the medical attendant told me he suspected typhoid fever; also that he understood there were other suspicious cases there not under his care. The patient was removed to Glenlivet and duly certified. The medical attendant himself contracted the disease. Between hearing of the case and receiving the certificate, a case of typhoid fever was notified from Tomintoul village. On enquiring into the matter, I found that the latter patient also had been in service at the farm above referred to and returned ill to Tomintoul. I also learned from his medical attendant that there was a case of typhoid fever at the farm which he was about to notify. On visiting the farm, I ascertained that the children (three) had been taken ill in March with what was supposed to be measles, and that the farmer himself was just convalescent

¹ Recently two outbreaks have occurred at the Asylum connected with these carriers.

from an attack of bilious fever. I suspected that the farmer and his children had been suffering from typhoid fever, and my suspicions were afterwards confirmed by a bacteriological examination. Another case occurred at the farm shortly afterwards, and two brothers of the Tomintoul patient contracted the disease. These three cases were removed to hospital, the last on the first of June. On July 9th, I received intimation of two other cases of typhoid in the village of Tomintoul in the cottage (semi-detached) adjacent to that in which the previous cases occurred. On visiting the following day, I found other members of the family were ill. They could not be removed to hospital owing to lack of accommodation. Arrangements were made locally. The disease was confined to the family. All the cases recovered.

A. P. came soon after to Aberdeenshire, and at the following places outbreaks of typhoid occurred while she was occupying the position of cook or cook-housekeeper:

1. Oct. 1901. Hotel, village of R.—four cases directly connected with the hotel.
 - (a) Maid servant, aet. 15. Notified October 21st.
 - (b) Daughter of the Proprietrix, aet. 7. Notified October 21st.
 - (c) Merchant's wife in village, aet. 25. Notified November 13th. Getting milk from hotel up to October 29th.
 - (d) The Proprietrix herself. Notified December 18th.

Other five cases of typhoid occurred in the village about this time, but had no known connection with the hotel.

2. Apr. 1902. Farm A. in the district of H.—three cases. The farmer, Mr G., his wife and daughter. All notified April 5th.
3. Jan. 1905. Farm O.—two cases. The farmer, aet. 50, and his son, aet. 16. Notified January 28th.
4. Aug.—Sept. 1907. Farm F.—four cases.
 - Aug. 31st. Farm servant, aet. 16.
 - 31st. Farm servant, aet. 25.
 - Sept. 5th. The farmer himself, aet. 40.
 - 29th. A daughter of the farmer, aet. 4.
5. Sept. 1908. Farm S., where as mentioned the farmer, Mr S., took typhoid and died.

In 1903, while A. P. was in the service of the manager of the G. Distillery in the district of H., he and his daughter each passed through a severe illness which, in the light of subsequent events, was most probably typhoid though not diagnosed as such at the time.

It will thus be seen that exclusive of the two suspicious cases in 1903, A. P. was responsible for 27 cases of typhoid occurring in six outbreaks.

On my recommendation, the Local Authority of the District in which Farm S. was situated resolved, in view of A. P.'s history and the fact that she had no means of livelihood if precluded from following her usual occupation, to offer her the equivalent of the Old Age Pension (she was then about 63), on condition that she engaged in no work that involved the handling of food. To this the Local Government Board gave their sanction. A. P. accepted the offer, and went to live in the Burgh of H. Here she resided alone for two years. I endeavoured, unfortunately without success, to obtain for her work of a kind that she could do without risk to others. As she had been accustomed to an active life, she tired of having nothing to do, and decided at the end of that time to give up her allowance and go back to work. She got an engagement in the neighbourhood. She was there, however, only a few weeks when

she again became insane and was removed to Banff Asylum. There the examination of her stools showed them to be still positive.

She recovered within a year and was discharged on March 6th, 1911. I obtained the sanction of the Committee to offer her her allowance again, but she refused it and fell back upon parochial relief. On November 12th, 1913, a case of typhoid was notified to me from a farm, three miles from the Burgh of H. where she was living. On enquiry, I found that A. P. had been temporarily engaged to help in the kitchen work. The patient, a farm servant, aet. 21, had a very severe attack. This was the last case with which A. P. was connected. She lived on in the Burgh of H. in receipt of parochial relief till November of 1918, when she died of acute bronchitis and asthma complicated with heart disease. To the end she remained convinced that she was not a typhoid carrier, and that she was the unfortunate victim of official interference which she very bitterly resented.

I may mention that this case is quoted (p. 35), by Ledingham and Arkwright(2), to whom I supplied the data there given.

Case 3. Miss S., aet. 53, sister of a farmer occupying Farm T.

An outbreak of typhoid at this farm in the autumn of 1908 led to Miss S. being identified as a carrier. Her history was as follows. Thirty-one years before, in 1877, she had an attack of typhoid fever. She was then 22 years of age. At that time, her mother being dead and her father having married again, she was living, with a brother, at a Farm G. in the vicinity of Aberdeen occupied by her grandfather, Mr R. Mr R. was a dairy farmer. Her grandmother, Mrs R., whom she helped with the work of the house, was not in good health and died shortly after. Miss S. then kept her grandfather's house up to the time of his death which occurred in 1889. Miss S.'s brother succeeded to the farm, and he and Miss S. continued to dairy till 1899, when they removed to a farm in Kincardineshire, W. M. There they remained till 1907. In that year they left W. M. and went to Farm T. in Aberdeenshire.

In the course of the 31 years from 1877 to 1908, no fewer than 24 cases of typhoid fever had occurred at the three farms of G., W. M. and T., among the servants, and in addition to these 24 cases, Miss S.'s brother, as also a nephew who stayed at G. and worked on the farm, had been attacked, making a total of 26 cases of definite typhoid, apart from some illnesses at G. diagnosed as influenza, but which were most probably cases of typhoid. As will be noted from the Table, two cases of typhoid also occurred among near neighbours while they were at W. M., probably from infection from Miss S.

The following Table gives a list of these cases with dates and details furnished to me by Miss S. and her brother. For particulars of the cases at W. M., I am indebted to Dr Macnaughton, County Medical Officer. I may mention that Miss S. assured me that no case of typhoid fever had occurred at G., or been associated with G., before her attack.

*Typhoid Carriers in Aberdeenshire**Farm G. 1877-1899.*

Miss S.'s attack was in July of 1877.

(R.I. = Royal Infirmary; C.H. = City Hospital; D.H. = District Hospital.)

- | | | | |
|-----|-------|------------|---|
| 1. | 1877. | Sept. | Servant girl. |
| 2. | " | " | Miss S.'s brother. |
| 3. | " | Nov. | Grieve on farm. Very severe attack. |
| 4. | 1878. | July. | Servant girl. Mild case. |
| 5. | " | Aug. | Farm servant. Removed to R.I. Mild case. |
| 6. | 1882. | Spring. | Servant lad. Removed to R.I. Very severe. |
| 7. | 1883. | Summer. | Cousin—young lad aet. 21—working on farm. Very severe. |
| 8. | 1885. | " | Kitchen maid, aet. 20. Mild. |
| 9. | " | " | Milk boy, aet. 15. Removed to R.I. Severe. |
| 10. | " | " | Man servant, aet. 22. Removed to R.I. Severe. |
| | 1893. | — | Two severe cases of "influenza" in this year, one of them fatal after a short illness, were probably cases of typhoid. Other cases of "influenza," according to Miss S., occurred at G. between the years 1892 and 1897 and were also possibly typhoid. |
| 11. | 1897. | Aug. 9th. | Farm servant. Removed to R.I. Very severe. |
| 12. | " | " 11th. | Farm servant. Removed to R.I. |
| 13. | 1898. | July 21st. | Farm servant, aet. 16. Removed to R.I. Died. |
| 14. | " | Nov. 18th. | Domestic servant. Went home ill to her mother's house in Aberdeen—found there to be suffering from typhoid. Removed to R.I. Died. |
| 15. | " | Dec. 2nd. | Farm servant. Removed to R.I. |

Farm W. M., Kincardineshire, 1899-1907.

- | | | | |
|-----|-------|-------------|--|
| 16. | 1900. | Aug. 26th. | Farm servant, aet. 23. Too ill for removal. Treated in farm house. Died. |
| 17. | 1901. | Jan. 29th. | Farm servant, aet. 22. Removed to C.H. |
| 18. | " | Mar. 16th. | Farm servant. Removed to C.H. |
| 19. | 1902. | Sept. 6th. | Farm servant, aet. 20. Removed to C.H.
(This month, September 1902, two near neighbours, I. N. and Mrs G., had very severe attacks of typhoid.) |
| 20. | 1903. | Sept. 11th. | Female servant, aet. 22. Removed to County Hospital. |
| 21. | 1904. | Jan. 25th. | Farm servant, aet. 18. Removed to County Hospital. |
| 22. | " | Mar. 16th. | Female servant, aet. 18. Removed to County Hospital. |
| 23. | 1905. | Sept. 5th. | Farm servant. Removed to R.I. as suffering from pneumonia. Found to be typhoid and removed to C.H. |

Farm T., 1907-1908.

- | | | | |
|-----|-------|------------|---|
| 24. | 1908. | July 24th. | Domestic servant. Removed to D.H. Died. |
| 25. | " | Aug. 28th. | Farm servant, aet. 46. Removed to D.H. |
| 26. | " | Oct. 13th. | Farm servant, aet. 40. Removed to D.H. |

Outbreak in Aberdeen through milk from G.—26 cases.

In 1898, Miss S. was associated through their milk supply with an outbreak of typhoid in Aberdeen in which 26 cases occurred. The outbreak commenced in November of 1898 and continued into January of 1899. It was found to be connected with a dairy R. in W. G. Street—to which Miss S. supplied milk, and suspicion was directed to Miss S.'s milk through the fact that a domestic servant, who had gone home from G. to her mother's house in Aberdeen ill, was found to be suffering from typhoid fever and removed to the Royal Infirmary (case 14 of Table). The beginning of this outbreak is thus described by Dr Hay in his *Report* for November 1898:

At the moment of writing, a third milk epidemic of typhoid appears to be commencing. To-day (Thursday) three cases, occurring in three quite separate families in different parts of the town and in no way associated with the other outbreaks, have been reported, and they all have the same milk supply. These cases, taken along with other two reported early in the week which also had been obtaining a part of their milk from the same dairy, make it

only too probable that the milk of this dairy also is infected. Enquiry was at once made into the source of the infection, but this is attended with much more difficulty than in the preceding outbreaks, owing to the dairyman, who has a business of considerable extent, deriving his supplies from a large number of farmers. We have, however, we believe, succeeded in tracing it to a farm in the Newhills District outside the City boundary from which a case of illness which was found to be typhoid, and which has indeed terminated fatally, was removed to the Royal Infirmary during the present month. We hope to be able to arrange with the help of the County Authorities for the immediate stoppage of this milk supply to any part of the City until these authorities are satisfied that the risk of the infection of the milk has entirely ceased. Meanwhile, a dairyman in the City, who has been obtaining a portion of his milk supply from this source, has undertaken to stop it forthwith. Owing, however, to the lengthened period of incubation of typhoid fever, it is likely that several cases, perhaps numerous cases, from this source will continue to develop themselves during the next two or three weeks.

As mentioned above, 26 cases in all, infected by milk from R.'s dairy, occurred before the outbreak stopped.

With Dr Hay's permission, I made an examination of the City notification Registers, and found that from 1891 to 1899, inclusive of the 26 cases in this outbreak, a total of 41 cases of typhoid had been notified among the customers of R.'s dairy in W. G. Street, and Miss S. informed me that during all these years this dairy received milk from G. When they went to Kincardineshire Miss S. ceased to supply milk to R., but for two years sent milk to a dairy W. in Aberdeen. I found from the notification registers that in these two years 15 cases of typhoid occurred among the customers of this dairy while only 6 cases occurred in the next seven years. This, of course, might have been merely a coincidence.

It seems not improbable that the milk from G. caused typhoid fever in Aberdeen at an earlier date. Miss S. and her brother told me that in 1885, Professor Simpson, at that time Medical Officer of Health for the City, "visited the farm in connection with typhoid," and that he had the water supply, which was derived from a pump near to the dungstead, analysed. It was found, however, satisfactory. It will be seen from the Table that in that year three cases of typhoid occurred at G., two were removed to the Royal Infirmary, and there may have been cases in Aberdeen connected with G. In a letter, in reply to an enquiry, Professor Simpson says: "The name G., Newhills, is familiar to me, and I am almost sure that one of the outbreaks was traceable to this farm." The late Mr Reid, Sanitary Inspector for the Aberdeen District, told me that he remembered on more than one occasion accompanying Professor Simpson to G. in connection with typhoid fever in the City after he joined the City staff in 1883.

The first time Farm G. was visited in connection with typhoid was in 1878. In that year Dr Burr and Dr Blaikie Smith were sent by the Parish Council of Aberdeen to investigate, because the Governor of the Poorhouse, which received its milk supply from G., reported that there was an outbreak of typhoid fever at the farm (cases 1, 2, 3 of the Table). The sanitary condition of the premises was found to be bad and Mr R. was warned. I could not

find, however, from the Parish Council records of that date any indication that cases of typhoid had occurred among the inmates of the Poorhouse.

It may be mentioned that both at G. and at W. M. every effort had been made to put the farms into good sanitary condition, no less than £300 having been spent at the latter farm on water, drainage, and a new sleeping-place for the men servants. Miss S. assured me that they left this latter farm "just to get away from the typhoid," and that they had gone to Farm T. "because cases of typhoid had never been known to have occurred there."

On learning that she herself had been the source of all the outbreaks, Miss S. was eager to know whether there was any cure for her condition. She decided finally to consult a surgeon in Aberdeen. The surgeon whom she consulted found that she was suffering from gall-stones and recommended an operation. The operation was carried out and two large gall-stones were removed. Typhoid bacilli were found in the bile and in the centre of one of the gall-stones examined. Miss S. proved to be suffering from tubercle, both of lungs and abdomen, and survived the operation only a month.

Miss S. was convinced that her infection came from a Farm W., three or four miles distant from G., and that it was brought to her by a maid-servant who came from W. to G. in 1877, and who had shortly before passed through an attack of typhoid fever there. All the maids at this farm, according to Miss S., got typhoid fever, the cause being put down to a "bad drain." The tenant of W. at that time was a Mr D. I have since ascertained that he and four members of his family suffered from typhoid in 1862, the first to take it being a daughter who had been visiting a house in the vicinity where cases of typhoid were. From this date to 1879 when Mr D. left the farm, outbreaks of typhoid repeatedly recurred at W., the maid-servants as mentioned above being chiefly attacked. It is significant that when Mr D. removed in 1879 to another farm an outbreak occurred there soon afterwards. Mr D. was married three times and cases occurred also among children of the third family, one of the daughters of this family having two attacks. The history clearly suggests that Mr D. had become a carrier from his attack in 1862, and was the source of the outbreaks at Farm W. and so possibly indirectly of Miss S.'s attack.

Case 4. Mrs B., aet. 28, wife of a cottar at Farm T.

Three cases of typhoid fever occurred in her house and the house adjoining in August and September of 1908. The first case was that of a man lodging in the adjoining house who was notified on August 26th. A child of Mrs B. was notified on September 14th, and her husband on September 24th. Mrs B. had passed through an attack of typhoid fever before her marriage when she was a servant at Farm S. where the series of cases occurred described on p. 434. Samples of her stools and urine were obtained and submitted for examination. The stools proved positive. With regard to the first of the three cases there was no evidence that the man had partaken of food in

Mrs B.'s house. Both houses, however, were served by one privy, and the midden connected with it was undrained and in a very filthy condition.

I lost sight of Mrs B. subsequent to these cases till 1921. When investigating the cause of a case of typhoid in the village of N. P. in that year, I found that she was living in the house adjoining that in which the case had occurred. She was now a widow, and had come to this house with her family a few months previously from Aberdeen where she had been resident for six years. The two houses were served by two privies which had a midden between them common to both privies, and the mother of the child with typhoid had to cross this midden when cleaning out her privy. The midden was in a very filthy condition. I obtained a sample of stools and urine from Mrs B., but they were negative.

1910.

Case 5. Mrs R., wife of a manufacturer, living at M.

There had been a number of outbreaks of typhoid at M. of which the following is a list subsequent to notification:

- 1894. June 16th. Domestic servant, aet. 20.
July 31st. Boy (son of employee), aet. 8. Died.
- 1896. Aug. 16th. Domestic servant, aet. 24.
Sept. 1st. Mrs S. (wife of employee), aet. 27.
10th. Domestic servant of Mrs S.
- 1906. June 15th. Domestic servant. Died.
Dec. 24th. Employee, aet. 17.
- 1907. Feb. 14th. Mrs C., aet. 24, sister of last case who stayed with her.
19th. Sister of Mrs C., aet. 13.
- 1908. July 20th. Man working temporarily at M., aet. 29.
- 1910. Sept. 29th. Case in house near, lad of 19, connection not very definite.

Mrs R. had an attack of typhoid in 1881. At that time a sister who came to nurse her and a maid developed typhoid, as did also a sister-in-law who lived next door and her maid. The local medical practitioner informed me in 1894 that "Mrs R.'s house for the last fifteen years had been periodically visited by typhoid fever." Everything had been done at M. in the way of sanitary improvements to try and stop the recurring outbreaks, but without avail.

While investigating the case in 1910, I obtained samples of stools and urine from Mrs R. whom I strongly suspected of being a carrier. Both stools and urine proved positive—the only case of a double carrier I have found. Strict precautions were enjoined and observed, but in spite of these another case, that of a maid-servant, occurred in September of 1912. It was then decided to inoculate the maid-servants, and this was always done up to Mrs R.'s death in 1922. No case subsequently occurred at M. or was traceable to Mrs R.

Case 6. Mrs M., wife of farmer occupying Farm C.

The following outbreaks were associated with her:

- 1904. Sept. 18th. Domestic servant, aet. 22.
Nov. 29th. Mrs M., a neighbour, aet. 35, getting milk from C.
- 1906. June 15th. Farm servant, aet. 18.
- 1907. Dec. 27th. Farm servant.
- 1910. June 14th. Farm servant, aet. 23.
Aug. 15th. Domestic servant, aet. 19.
Nov. 9th. Mrs T., a neighbour, aet. 43, supplied with milk from C.

Mr M. himself had an illness in 1909 which was diagnosed as influenza, but which might have been typhoid. He was confined to bed for three weeks, and, after a relapse, for three weeks more, insomnia being a marked feature of the illness. A prolonged illness of one of the children was probably also typhoid.

Mrs M. had passed through an attack of typhoid in 1903. The examination of the stools and urine in connection with the outbreak in 1910 showed her to be a faecal carrier. No further case occurred at C. till 1917 when a domestic servant, aet. 18, developed typhoid. This case presented certain interesting features. She was thought at first to be threatening an attack of appendicitis and went home. She was sent to the Royal Infirmary for operation by her own medical attendant. The examination made there suggested the possibility of typhoid, and the blood was examined with positive result. She was accordingly removed to the City Hospital. The symptoms of appendicitis, however, became more pronounced and she was operated on. She proved to be suffering from both diseases, but made a good recovery.

An examination of Mrs M.'s stools in 1921 showed her to be still a carrier. A positive result was not obtained till the sixth sample was examined.

The treatment of this case with detoxicated vaccine is described in a paper in the *Lancet*, 1923, i. 378. I may mention that the non-agglutinating bacillus referred to in that paper reappeared, and that an extensive investigation which Professor Carl Browning of Glasgow University very kindly made of it showed that it was a true *Bacillus typhosus* with deficient agglutinability. A course of vaccine prepared from this bacillus itself was then tried. The results of this treatment will be given later when more samples have been examined, but the results so far seem interesting.

1911.

Case 7. Mrs R., wife of crofter and labourer, carrying on a small dairy business in the village of M.

The following cases were associated with her through the milk supply:

1902.	June 30th.	Male,	aet. 43.	
1907.	Mar. 19th.	Female,	„ 7.	
	Apr. 9th.	Male,	„ 10.	Died.
1908.	Aug. 19th.	Female,	„	
1910.	Aug. 8th.	„	„	—
1911.	June 7th.	Male,	„ 47.	
	7th.	„	„ 14.	
	27th.	„	„ 14.	

It was in connection with this last outbreak that I obtained samples of stools and urine from Mrs R. who had passed through an attack of typhoid earlier in her life. The examination of the stools showed her to be a faecal carrier. Mrs R. undertook to give up all personal connection with the dairying work, and engaged a woman to milk and do everything in connection with the dairy. Her daughter later took this woman's place. The dairy is now closed.

Case 8. Mrs W., wife of farmer, occupying Farm A.

In the latter part of 1911 and the beginning of 1912, the following cases of typhoid were notified from this farm:

1911.	Sept.	3rd.	Servant girl, aet. 14.
	Dec.	2nd.	Mrs S. (monthly nurse attending on Mrs W.), aet. 45.
		19th.	Domestic servant, aet. 31.
		21st.	Stepson of Mrs W., aet. 9.
1912	Jan.	21st.	Farm servant, aet. 17.

I obtained samples of stools and urine from all the adults on the farm. The stools of Mrs W. proved positive. I did not obtain a history of her having had an attack of typhoid, but there had been typhoid fever at the farm occupied by her father when she was a girl, and it is possible that she may have had a mild and unrecognised attack then.

In February of 1913 another case was notified from A., a domestic servant. Then a long interval elapsed without any further outbreak till 1921, when a domestic servant was removed to hospital suffering from typhoid. The case was a very mild one. I obtained two samples of stools and urine from Mrs W. but both were negative.

A further outbreak occurred in connection with A. in the end of 1922 and beginning of 1923. Two servants (M. and F.) left the farm ill and one of them caused two cases before the nature of his illness was recognised. Eight samples of stools from Mrs W., eight from the stepson who had typhoid in 1911 and two from Mr W. have been examined since, but all have been negative.

Case 9. Mrs R., wife of labourer, in the village of S., associated with one case of typhoid in 1905, and with another case in 1911.

A sample of stools examined in connection with the case in 1911 showed Mrs R. to be a faecal carrier. No cases of typhoid have since been traceable to, or associated with her.

1916.

Case 10. Mrs McR., aet. 45, widow, acting as temporary cook to Miss W., tenant of Farm C.

In 1916, an outbreak of typhoid fever with five cases occurred at or in connection with this farm. The first case was reported from Edinburgh, a girl of 14, who had been staying on holiday at a house near Farm C., the occupants of which got milk from C. The case was reported on September 25th, while Miss W., aet. 61, the tenant of C., who had been ill for some time, was notified on September 27th. Mrs B., in whose house the case notified from Edinburgh had been living, fell ill and was notified on September 30th. Two more cases occurred in October, one a lad of 17, son of Mrs B., and the other a niece of Miss W., aet. 44, who had come to look after the house. Miss W. was being treated at home. The following table shows the cases with the dates of notification:

Sept.	23rd.	Girl, Edinburgh case, aet. 14.
	27th.	Miss W., occupant of farm, aet. 61.
	30th.	Mrs B., neighbour getting milk, aet. 53.
Oct.	17th.	Son of Mrs B., aet. 17.
	17th.	Miss M., niece of Miss W., aet. 44.

I succeeded in obtaining samples of stools and urine from the two domestic servants employed at C. The stools of the cook, Mrs McR., proved positive. I could get no history of her having had an attack of typhoid or any previous association with typhoid, though, according to her statement, she had milked cows and had been engaged in work likely to spread infection. Her home was in Aberdeen, and she had been temporarily engaged as cook by Miss W. She returned to Aberdeen soon after. Dr Hay, to whom I communicated the circumstances of the outbreak, persuaded her to go to the City Hospital for treatment. After six weeks' stay there, as the stools became negative, she was discharged. It should be mentioned that while she was at C. she assisted in milking the cows.

In January, 1924, Mrs McR. has again been associated with an outbreak of enteric fever—three cases having occurred in a family related to her with whom she was staying, the first case having sickened fourteen days after her arrival. Typhoid fever in this family was confirmed bacteriologically but the examination of two samples of faeces and urine from Mrs McR. gave a negative result.

1917.

Case 11. Mrs T., widow, aet. 65, living alone in the village of P., and doing such work as nursing, milking, etc.

In 1917, an outbreak of typhoid occurred in the village of P. with the following cases:

June	19th.	Male,	aet.	10.
	28th.	Female,	"	8.
	30th.	"	"	35.
July	4th.	Male,	"	6.
	6th.	Female,	"	69.
	7th.	"	"	23.
	14th.	"	"	37.
	15th.	Male,	"	7.
	19th.	"	"	3.

The cases occurred in five households which got their milk from a croft where one cow was kept and the surplus milk sold. The milking was done by the sister of the tenant of the croft and by Mrs T. I obtained samples of stools and urine from both. There was no history of either having passed through an attack of typhoid. The first two sets of samples were reported negative, but a third set which, with some difficulty, I succeeded in obtaining, I had examined in two laboratories and this examination showed Mrs T. to be a faecal carrier. This result was subsequently confirmed by a number of examinations of her stools. As stated she had no history of ever having passed through an attack of typhoid or of ever before having any association with typhoid. She had had an attack of influenza in the winter of 1916-17, but the symptoms were very indefinite.

In November of this same year (1917), Mrs T. went to keep house for a friend for a few days when the latter was away on holiday, and five weeks later a lodger in the house, a lad of 20, was removed to hospital suffering from typhoid.

As Mrs T. had no adequate means of livelihood without doing work which involved the handling of food, the District Committee agreed, on my recommendation, to make her an allowance of 10s. a week on condition of her abstaining from any kind of work connected with food or with nursing. This she accepted, and drew the allowance till her death which took place in 1920.

1918.

Case 12. Mr M., tenant of Farm N.

The following outbreaks had been associated with this farm during the tenancy of Mr M.:

- 1901. June 6th. Farm servant, aet. 18. (This case went home ill and infected several members of his family.)
- 1915. — Son, aet. 8.
- 1916. — Farm servant, aet. 16.
- 1917. Nov. 29th. Farm servant, aet. 42.
- Dec. 1st. Farm servant, aet. 16.

In connection with the outbreaks in 1916 and 1917, samples of stools and urine from Mr M. and his wife and son, were examined—one set in 1916 and two in 1917—but were reported negative. In 1918, I obtained other samples although no other outbreak had occurred in the interval as I felt convinced that Mr M. was a carrier. He was not married in 1901 when the first outbreak occurred and he alone therefore had been at the farm on the occasion of all the outbreaks. As I anticipated, the stools of Mr M. proved positive. No further case occurred at N. till 1922 when a maid-servant became ill, went to her home in Aberdeen where her illness was found to be typhoid and she was removed to hospital. I failed to obtain samples of stools from Mr M. in connection with this last case, but the examination of a sample of stools in connection with another case which occurred in 1923 showed that Mr M. was still a carrier.

Case 13. Mr S., crofter, occupying croft of B. of A.

Mr S. worked in his spare time on farms in the neighbourhood, and it was observed that he had been working at three places at the time when cases of typhoid had occurred there. He was in this way associated with the following outbreaks:

- 1912. Farm A.—four cases.
 - Aug. 9th. Farm servant, aet. 30.
 - Sept. 20th. Son of previous case, aet. 4.
 - Oct. 18th. Farm servant, aet. 16.
 - 18th Domestic servant, aet. 16.
- 1914. Farm B.—five cases.
 - Oct. 27th. Farm servant, aet. 24.
 - 28th. Farm servant, aet. 29.
 - 31st. Farmer, aet. 38.
 - Nov. 26th. Female servant, aet. 27.
 - 30th. Female servant.
- 1917. Farm C.—one case.
 - Sept. 21st. Female, aet. 50.

For this last case I could find no possible cause apart from the fact that Mr S. whom I knew to have had an attack of typhoid a number of years before, had been working at the place for some time and would have been there at the probable date of infection. I obtained two samples of his stools and urine and they were examined, one on November 12th and the other on November 24th, 1917, but both were reported negative. I retained the conviction, however, that Mr S. was a carrier, and in September, 1918, though no fresh cases had been associated with him, I obtained further samples from him as also from his wife and daughter, who also had had typhoid fever at the time of Mr S.'s attack, and had them examined in two laboratories. The stools from Mr S. proved positive. No further case was associated with him till 1922 when a boy, a grand-nephew, who had been on a visit to Mr S. developed typhoid immediately after his return home. I asked for but did not obtain further samples of stools from Mr S. An outbreak with four cases at a farm adjoining in 1923 was almost certainly connected with Mr S. Samples of stools were again refused.

In illustration of this last case, it may be worth while giving the following history of a farm servant who was associated with four outbreaks of typhoid at farms on which he was working when the outbreaks occurred, but who refused to give me samples of his stools and urine so that I could not definitely determine whether he was a carrier or not. His history certainly pointed very strongly to his being a carrier. It was as follows:

This man (U.) had an attack of typhoid in 1892 and in 1900 two of his sons, in 1907 a daughter (2½ years), and in 1909 another daughter (7 years) all had attacks of typhoid fever. U. was working as a farm servant on the following farms when outbreaks occurred:

- 1905. Farm A.
 Sept. 1st. Daughter of farmer, aet. 23. Died.
 Dec. 31st. Boy, child of cottar, aet. 11.
- 1906. Jan. 21st. Daughter of farmer, aet. 19.
 21st. Son of farmer.
 27th. Domestic servant.
- 1907. Farm L.
 Jan. 29th. Farm servant, aet. 33.
 Feb. 7th. Wife of last case.
 Apr. 15th. Child of U, (cottar house), aet. 2½.
- 1909. Farm A:
 May 15th. Farm servant, aet. 20.
 July 24th. Farm servant.
 Aug. 14th. Farm servant.
- 1911. Farm E. C.
 Sept. 10th. Farm servant, aet. 17.
 20th. Son of farmer, aet. 21.
 20th. Daughter of farmer, aet. 15.

In connection with the outbreak at E. C. in 1911, I used my utmost efforts to obtain samples of stools and urine from U., but his wife persuaded him not to grant my request.

Case 14. Mr L., aet. 67, retired.

Mr L. had an ambulant attack of typhoid in 1918. He was never confined to bed, but his blood and stools were positive. His wife had an attack at the same time which proved fatal. Mr L.'s stools continued positive for a number of months and were still positive when the examinations were discontinued. No case of typhoid, however, has been associated with him.

In 1922, in connection with an outbreak of what proved to be para-typhoid fever in a house near by, I took the occasion to have a series of samples of Mr L.'s stools examined. All proved to be negative.

The infection to Mr L. and his wife came from a neighbouring farm through the milk which was the source of the epidemic in Aberdeen in 1918.

Case 15. Mrs P., aet. 33, residing in the village of P.

Mrs P. had an attack of typhoid in 1917 along with two of her children. She was treated in hospital and discharged only after several examinations of her stools and urine had shown them to be negative. In the following year another child of hers was notified on September 21st and another on November 7th as cases of typhoid. There was a possible source of infection through the milk from a case which had occurred at the dairy where Mrs P. got her milk supply. In view, however, of Mrs P.'s attack in the previous year, I thought it well to have a sample of her stools and urine again examined. The stools proved positive. She has been connected with no case since nor has any further sample been examined.

Case 16. A. B., aet. 45, servant at a dairy farm.

A. B. had conducted the dairy operations at Farm F. for a number of years. She had been connected with several cases. A servant girl at the farm had an attack of typhoid in November, 1896, while the tenant of the farm, her first master, died of typhoid in 1909. I endeavoured in 1908 to obtain samples of her stools and urine, but without success. In connection, however, with a case which occurred in 1918 among the customers of the dairy, I succeeded in obtaining samples from her of stools and urine, as also from her master and mistress, both of whom had had typhoid fever. The first two sets of samples were reported negative, but a third set, which I obtained and had examined in two laboratories, showed that A. B. was a faecal carrier. She was immediately removed from all connection with the milk and every precaution taken. No case was subsequently connected with the dairy, which has since been closed, or with her.

In 1920, A. B. attended the City Hospital in Aberdeen for treatment. There her stools were found to give almost pure cultures of typhoid bacilli.

1919.

Case 17. Mrs B., crofter's wife, aet. about 60.

Case 18. Mr R., crofter, aet. about 75.

Case 19. Mrs R., wife of above, aet. about 70.

These three carriers were identified at the same time through an outbreak of typhoid in 1919. Their two crofts were within half a mile of each other. The circumstances of this outbreak were as follows: On May 22nd, two children, brother and sister, age 13½ and 12, were notified from a house in the vicinity of these crofts as cases of typhoid. Their mother had been for six weeks previously nursing a man who had been suffering from what had been diagnosed as an attack of influenza. This man was a son-in-law of Mrs B. and worked on her croft, but had a home of his own. I had long suspected Mrs B. of being a carrier from outbreaks with which I knew her to have been associated, and her history suggested that this illness of her son-in-law might really be typhoid. I communicated my suspicion to his medical attendant who had a sample of blood taken and examined. The result of the Widal test was positive. Before the contacts could be inoculated, a grand-daughter of Mrs B., a girl of 17, who was acting as a servant to her, was notified from her home, whither she had gone ill, as a case of typhoid. In the course of the investigations, I found that the mother of the two children, first notified, obtained her milk from Mrs R. I knew that Mrs R. also had been associated with two outbreaks of typhoid, and as she and her husband had both had typhoid, as also had Mrs B., I asked for and obtained samples of stools and urine from all of them. The examination showed all three to be faecal carriers. There were therefore two sources from which the children might have received infection, but it was most probably, I think, carried by their mother from Mrs B.'s son-in-law while she was engaged in nursing him.

I may add that the district of M. in which these crofts were situated was in former years noted for its typhoid incidence.

The following were the outbreaks with which Mrs B. and Mrs R. had been previously connected:

Mrs B.

1892.	Aug.	26th.	Female, married.	Living in same house as Mrs B.
		27th.	Male, husband of above.	
1893.	Mar.	17th.	Female, aet. 13.	Milk from Mrs B.
	May	26th.	Male, aet. 29.	" "
1897.	July	19th.	Male, adult.	" "
1898.	Sept.	3rd.	Male, adult.	" "
1901.	May	15th.	Female, adult.	" "
1904.	Oct.	10th.	Male, adult.	" "

Mrs R.

1893.	June	24th.	Male, aet. 52.	Milk from Mrs R.
		24th.	Female, aet. 10.	" "
		25th.	Male, Mrs R.'s husband, aet. 46.	" "
		25th.	Female, Mrs R.'s daughter, aet. 16.	" "
		25th.	Female, girl living in house, aet. 12.	" "
1903.	Oct.	28th.	Male, farm servant, aet. 18.	" "

Cases 20 and 21. Mr R., farm servant, and his wife.

These two carriers were found in 1919 in connection with an outbreak at a farm where four cases occurred and where the man was a farm servant.

He and his wife had passed through an illness two years before which was almost certainly typhoid though not diagnosed as such at the time. Both were found to be faecal carriers.

Case 22. Mrs B., aet. 70, widow, living with two grandchildren in the village of C.

In 1918-19 the following outbreak of typhoid occurred in this village:

1918.	Sept. 27th	Female, aet 44.
	Nov. 13th.	" " 13.
	16th.	" " 5.
	22nd.	" " 2.
1919.	Jan. 3rd.	" " 8.
	17th.	" " 9.
	18th.	" " 6.

The source of the outbreak was for a time very difficult to ascertain. No connection through milk could be found between the cases. Samples were obtained from all the milkers involved in the different supplies, but they proved negative. Later, information was given to me by the mother of two of the cases that the two grand-children of Mrs B., who lived close to her, had had prolonged illnesses in August and September, 1918, which were unattended by a doctor. I found on enquiry that the symptoms in these cases pointed to typhoid, and this was fully confirmed by the examination of samples of blood from each of the two children, the blood giving strongly positive Widal reactions. They had really been the first cases in the outbreak, and the other cases could be linked up with them. The grandmother had had a very severe attack of typhoid seven years before during the epidemic in Peterhead where they were then living, as also had her husband and three of the family, one of the family dying. Mrs B. readily agreed to give me samples of stools and urine and the examination of these proved her to be a faecal carrier. She had only come to the village of C. six months before, having lived from the time of her attack up to that date in the town above mentioned, but she had not been associated there with any outbreak of typhoid.

I may add that nearly all the children in the village and some of the adults, about 85 in all, were inoculated as a preventive measure before the discovery of Mrs B. as a carrier. Mrs B. has since died.

It may be worth recording here the facts regarding two farms in the county at which the occurrence of outbreaks over a long series of years pointed clearly to the presence of carriers. One of these was Farm B. in the A. District, occupied by Mr R. Here for thirty years, prior to 1894, outbreaks of typhoid had occurred so frequently that in the district the illness came to be known as the "B" fever. As happens in such cases, it was the new servants chiefly that took the disease. Many of them, going home ill, carried infection to their families. The number of cases of typhoid directly or indirectly connected with B. was put in the district as high as 50-60. The last case occurred in February, 1893, a servant girl, 15 years of age. A new water supply had been introduced

at the farm a number of years before and the drainage entirely renewed, but this had had no effect upon the incidence.

In 1894, Mr R.'s son succeeded to the farm. No case occurred after Mr and Mrs R. left. I learned recently that Mrs R. had an attack of typhoid fever when she was a girl, and at that time there was a serious outbreak at her father's farm. All the circumstances point to her having been the carrier.

The other case to which I would refer was Farm S. in the D. District. From 1896 to 1906, there had been recurring outbreaks at this farm, as the following table will show:

1896.	Oct.	1st.	Farm servant,	act	28.
		1st.	" "	"	16.
		1st.	Domestic servant,	"	—
		7th.	Farm servant,	"	16.
		7th.	" "	"	21.
1897.	Sept.	9th.	" "	"	19.
1900.	Aug.	6th.	" "	"	18.
	Oct.	22nd.	" "	"	17.
		30th.	Boy in cottar house,	"	6.
1901.	Feb.	25th.	Farm servant,	"	22.
	Aug.	28th.	" "	"	—
1906.	June	3rd.	" "	"	24.
	Aug.	17th.	Domestic servant*,	"	25.
		25th.	Farm servant.		

* *This case is carrier No. 4.*

The carrier at S. was most probably the housekeeper, Miss G. Mr P., the tenant of the farm, was unmarried. Farm S. was not the only place where Miss G. was associated with typhoid. Cases of enteric had occurred at Farm L. which Mr P. had occupied before going to S., and where also Miss G. acted as his housekeeper. After Mr P.'s death, Miss G. left S. and went to reside at C. There in 1910, enteric fever broke out in a family living in a house adjoining. One of the children of this family, 3½ years old, who was in the way of going into Miss G.'s house and getting food from her, developed typhoid and was notified on October 27th. Two more cases occurred in the house from which the first case, which had not been removed to hospital, was notified, and were reported on November 29th and December 8th. On this occasion I obtained a sample of Miss G.'s stools and urine, but they proved negative, and further samples were refused. One negative result is, of course, inconclusive. Miss G. died some time later, but no cases were associated with her subsequent to 1910, and no further cases have arisen among those closely associated with her during her life.

POSITION OF THE LAW IN SCOTLAND IN REGARD TO TYPHOID CARRIERS.

Up to 1919, there was no legal recognition of a carrier. In the Public Health (Pneumonia, Dysentery, etc.) Regulations issued in that year, carriers of dysentery were recognised and certain powers granted in regard to "such as were concerned with the preparation or handling of food or drink for human consumption." By Article 14 of the Regulations, these powers were extended

to embrace typhoid carriers. "The responsible manager of the trade or business is to afford the medical officer of health all reasonable assistance in getting a clinical examination of such person carried out." It does not say what is to be done if the person suspected refused to give the samples of stools and urine necessary to prove whether they are carriers or not. There seems no power to compel them to do so though a suspicion or a reason to believe that a person was a carrier would probably be sufficient to exclude him or her from the work of handling food.

By the Public Health (Infectious Disease (Carriers) Regulations, issued in 1921, a Local Authority is given for three months the same powers over a carrier as are given by the Public Health Act over persons suffering from the disease. For the purposes of the Regulations, a person has to be certified as a carrier by the medical officer of health and one registered practitioner, and to be "a danger to others by reason of the probability of his spreading infectious disease." The certificate is to extend for three months, a further certificate to be granted on a re-examination. At any time during the currency of the certificate, the carrier can demand to be re-examined on giving to the medical officer of health of the area not less than 48 hours' previous notice in writing, and if as a result of any such re-examination the person is not certified as aforesaid, the Regulations shall cease to apply to and as respects him.

Two points do not seem to be adequately recognised by these Regulations, first, that, with few if any exceptions, a chronic typhoid carrier remains a carrier for life, and second, that owing to the intermittency of the discharge of typhoid bacilli in the stools, negative results would constantly be obtained and carriers cleared while they still remained dangerous foci of infection. They would not readily thereafter give further samples of stools or urine. Further, there is no power given for an examination of those who are not concerned with the preparation or handling of food or drink for human consumption.

I am strongly of opinion that power should be given to obtain samples of stools and urine compulsorily where these are refused and where the evidence is such as would justify a warrant being granted. Removal to hospital for a limited time might be required so as to ensure a proper examination. Refusal to grant samples has more than once interfered with the complete investigation of important epidemics, such for example as that in Aberdeen in 1912. Certain facts that came subsequently to light added probability to the source of this epidemic being infection of milk from a carrier. I discovered some years after that the woman suspected of being the carrier in 1912 was, in 1892, associated with an outbreak of typhoid—six cases in four families—in a small village to which at that time she supplied milk. Only one sample of stools and urine was obtained from her in 1912, and further samples were refused. The sample was negative, but one negative result is, of course, inconclusive. Dr Hay⁽⁴⁾, in his *Report* upon the epidemic, says that he had a personal interview with

the Medical Member of the Local Government Board as to whether the law gave power to obtain such samples compulsorily. The latter was inclined to think that the Public Health Act did give the necessary power, but admitted that, although cases presenting a similar difficulty had previously arisen, the Board had as yet not seen its way to advise the adoption of compulsory measures. Dr Hay adds, "it is doubtful if there is legal power to compel such examination."

I frequently have been refused samples where the evidence pointing to a certain person being a carrier and the cause of an outbreak seemed very strong. Such refusal of samples is not likely to grow less as powers over carriers are increased.

If, as the result of a bacteriological examination, a person is certified to be a carrier, and his or her living is interfered with, compensation should be given for any loss incurred thereby, and proper provision should be made, if required, for their maintenance. This expense should not devolve on Local Authorities, but should be borne, partly or mainly, by the State. One reason that may be noted in passing is that carriers are more likely to be identified in rural areas, and that too much of the burden would fall upon such areas, while the discovery and control of carriers is equally important for the towns in view of the danger of milk epidemics. The whole question is one that should be dealt with on the most comprehensive lines.

CONCLUSIONS.

1. That the chief source of typhoid fever and the sole cause of its remaining endemic in a rural area is the presence of carriers in that area.
2. That almost all these carriers are faecal.
3. That when the carrier condition becomes chronic, recovery seldom, if ever, takes place, as far as our present knowledge goes.
4. That the law dealing with typhoid carriers requires expansion of the powers conferred.
5. That proper provision should be made for the maintenance of typhoid carriers where their means of livelihood has been interfered with, and that this should be done mainly by the State.
6. That every effort should be made by the Government to encourage and co-ordinate work directed towards finding a cure.

The problem of the cure of carriers is perhaps the most important of all Public Health problems of the present time. As the *Lancet* puts it in a leader of November 13th, 1920: "The 'carrier' problem in infectious disease is one of the most difficult and, at the same time, one of the most urgent questions from the point of view of the hygienist, the bacteriologist, and the medical practitioner. It is incidentally one of great interest to the Public, although it may be doubted whether that interest has yet been sufficiently aroused.

Nor is the time quite ripe for insisting upon public education, since it must be confessed that efficient methods of discovering the carriers and of rendering them innocuous have yet to be evolved."

In conclusion, I take leave to say that the more my experience of administrative work in a rural county has been, the more am I convinced that rural areas offer opportunities for the elucidation of epidemiological problems such as cannot be got in crowded cities, and this holds more particularly in the case of typhoid and diphtheria.

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THE PULMONARY LESIONS PRODUCED BY THE INHALATION OF DUST IN GUINEA-PIGS.

A REPORT TO THE MEDICAL RESEARCH COUNCIL.

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(*From the Laboratory of Physiology and the Cherwell Laboratory.*)

(With Plates VI-X.)

CONTENTS.

	PAGE
1. Introductory	439
2. Literature	439
3. Material and methods	443
4. The histology of the normal guinea-pig lung	444
5. The lesions caused by the dusts used in these experiments	
(1) Mixtures of flint and coal (2 series)	445
(2) China clay (3 series)	447
(3) Felspar	448
(4) Ground pitcher	449
(5) Pure amorphous (i.e. precipitated) silica	450
(6) Pure flint	450
(7) Pure coal	451
(8) Pure shale	452
(9) Ignited shale	453
(10) Dried earth	453
(11) Ignited earth	454
(12) The bacteriological examination of the sections	454
6. Discussion of the results	
(1) Mixtures of flint and coal (2 series)	456
(2) China clay (2 series)	457
(3) Felspar	458
(4) Ground pitcher	459
(5) Pure amorphous silica	459
(6) Pure flint	461
(7) Pure coal	461
(8) Shale	462
(9) Ignited shale	462
(10) Dried earth	462
(11) Ignited earth	463
(12) The broncho-pneumonic lesions	463
7. The origin of the dust-cells	464
8. The mode of elimination of dust particles from the lungs	465
9. Summary	467
10. Appendix	468
Explanation of Plates	469
Bibliography	471

1. INTRODUCTORY.

THESE experiments on the lesions produced by the inhalation of certain dusts in guinea-pigs were undertaken on behalf of the Medical Research Council, which very generously provided the necessary funds and assistance.

Over 100 animals, including the controls, have been used in the course of these observations, extending over a period of two years, observations which form, in part, a sequel to the experiments of Mavrogordato (1918) also carried out on behalf of the Medical Research Council.

The object of this investigation was partly to complete certain of Mavrogordato's experiments, partly to examine the effects of various other dusts upon the lungs.

In addition, the controversial question of the origin of the dust-cells, and the modes of elimination of the dust particles from the lungs, have been carefully studied.

And, finally, evidence has been brought to bear upon the alleged relationship between the degree of harmfulness of a given dust and the degree of sharpness and of angularity of its constituent particles.

My acknowledgments are many. I am indebted to Dr J. S. Haldane for suggestions on many points. It is with pleasure that I take this opportunity of expressing to him my gratitude for many kindnesses. I have to thank Professor Sir Charles Sherrington for his continual interest in my work and for according me every facility in his laboratory. I am also deeply indebted to Dr A. G. Gibson for valuable comment on the lung lesions, while to the Managing Director, and to Dr White, of the Worcester Royal Porcelain Co., I must express my gratitude for information on the dusts used in connection with the manufacture of china. I am also indebted to this establishment for the benefit of a personal visit. And, finally, I have to thank Prof. Mellor for supplying certain of the dusts used in these experiments, and the Mining Research Laboratories of the University of Birmingham for igniting the samples of earth and shale.

I have to thank Mr F. Haynes for taking over the technical portions of this research and for doing a number of the autopsies, and Mr W. Chesterman of the Anatomical Department for his skilful microphotographic rendering of difficult material.

2. LITERATURE.

The bibliography of Dust-Inhalation is so vast that no useful purpose would be served by mentioning other than the more important memoirs bearing directly on the subject-matter of this paper. The memoirs are summarised in chronological order, further comment being reserved for the discussion on pp. 455 to 463.

Arnold, in 1885, published the most important of the early contributions. In this lengthy monograph, which contains no little repetition, Arnold brings out the following points:

The inhalation of soot, emery and sandstone particles by rabbits and dogs caused

definite lung changes. These comprised thickening and infiltration of the alveolar walls, processes so marked as sometimes to result in the formation of nearly solid areas of lung. Dust particles—both intra- and extra-cellular—were observed in the lung substance, in the bronchi, and in the bronchial glands.

From the inspection of the figures illustrating Arnold's sections, made, of course, at a time when staining methods were not in current use, one suspects that the chief lesions were those of an interstitial pneumonia with well-marked fibrosis. Arnold noted, furthermore, that some of the dust left the lungs and was deposited in the lymphoid tissue both beneath the bronchial epithelium and in the bronchial glands. Without committing himself definitely, he was of opinion that soot particles, at any rate, found their way from the alveoli into the lymphoid tissue *via* the lymphatics. In this way he accounted for the tendency for dust to disappear from the pulmonary alveoli in course of time. The large cells, often containing dust particles, which Arnold observed in the alveolar cavities, he regarded as being derived by proliferation from the alveolar epithelium.

Tchistovitch (1889) studied the phagocytosis of foreign bodies in the lungs with special reference to the origin of the cells which ingest them. He injected carmine particles into the trachea of frogs and guinea-pigs. He also caused the latter to inhale lamp-black in a special chamber.

Tchistovitch observed that phagocytosis occurred, in all these experiments, after a latent period, which, in the case of guinea-pigs exposed to fine soot particles, was approximately 14 hours. He claimed that the phagocytic cells were always macrophages originally derived from the leucocytes of the blood, and that the alveolar epithelium, though often covered with soot and other particles, neither ingested them nor ever showed signs of proliferation. In all these respects the views of Tchistovitch are diametrically opposed to those of Arnold.

Claisse and Josue (1897) repeated the lamp-black experiments just described on dogs, rabbits and guinea-pigs. They confirmed Arnold's view that dust particles tend to be eliminated from the lungs *via* the lymphatics to the bronchial glands and, ultimately, to the spleen. With regard to the effects produced by the dust they say: "*Il se produit donc, à la longue, chez nos animaux, une infiltration charbonneuse considérable des poumons et des ganglions bronchiques...Les poumons sont en pleine activité épithéliale,...mais nous n'avons pas constaté chez nos animaux purement anthracosiques de véritable réaction inflammatoire.*"

These authors further deny that siderosis produces lung lesions, which, they assert, are only caused by dusts composed of large and angular particles. The trauma caused by these to the bronchial epithelium results in infection by micro-organisms and consequent lesion to the adjacent lung substance.

Metchnikoff (1901) in accordance with his phagocytic theory of defence, strongly upholds the contention of his pupil Tchistovitch (*vide sup.*) that all the phagocytic cells found in the lung under inflammatory conditions are derived from the leucocytes of the blood.

Chantemesse and Podwysotsky (1901) claim that the removal of dust from the lung is largely due to leucocytes apparently derived from the blood.

Washbourne and Eyre (1902) note the presence of areas of collapse in the lungs of animals exposed to dust.

Dust particles within the alveoli are eliminated thus: "some pass through into the lymphatics and either reach the bronchial glands, where they are retained, or are retained in the adenoid tissues (peri-bronchial and peri-vascular) and thence may be excreted to the bronchi by means of cells, whilst some are taken up by cells and thus passed into the bronchi are ultimately extruded through the upper air passages."

Oliver (1903) concludes from the study of anthracosis among coal-miners, that a mild degree of pulmonary anthracosis is not inconsistent with health. He also considers that tubercle, when present in such cases "is an accidental infection."

Wainwright and Nichols (1905) made some remarkable observations on the protective effects of coal dust against infection by the tubercle bacillus. One group of guinea-pigs was exposed to coal dust for two months; the animals then received an intra-tracheal injection of a culture of tubercle bacilli. The animals of the other (control) group were not dusted, but received a similar injection. The animals exposed to the coal dust showed "extensive

T.B. of the abdominal viscera and of the glands around the tracheal region, but the lungs were free." The controls, on the other hand, were found to have "extensive T.B. of the lungs and abdominal viscera."

Briscoe (1908) exhaustively studied the effects of foreign bodies of various kinds—micro-organisms, red blood corpuscles, etc., but not dust particles—on the alveolar epithelium of guinea-pigs. He concludes that the alveolar epithelial cells undertake most of the phagocytosis in slight infections, while the polymorphonuclear leucocytes are the most actively phagocytic elements in acute infections. Regarding the origin of the dust, and other large phagocytic, cells of the lung, Briscoe is of opinion that they are derived from the alveolar epithelium by proliferation.

Beattie (1912) made experiments on the effects produced in guinea-pigs by the inhalation of the following dusts:

- (i) Coal.
- (ii) Shale (as used for stone dusting in collieries against explosions).
- (iii) Stone dust containing much silica.

From these experiments Beattie distinguishes between the dusts which are harmful—i.e. produce fibrosis—and those which are not. The dusts which cause fibrosis increase the susceptibility to infection. Beattie concludes that "the more irritating the dust the more intense the fibrosis."

Haythorn's (1913) observations on the reaction of the lungs of guinea-pigs to the inhalation of lamp-black leads him to the conclusion that the dust-cells are probably derived from Endothelial Leucocytes—i.e. Large Mononuclears and Transitionals. Haythorn is further of opinion that "...carbon pigment once taken up by the cells remains intra-cellular indefinitely unless freed by some process producing general necrosis of the tissues."

Haldane (1916) has furnished the following figures as a striking testimony of the relative rarity of phthisis among coal-miners:

*Death-rates from phthisis per 1000 living at each age-period
for England and Wales, 1901-1902.*

Age period	15-25	25-35	35-45	45-55	55-65
Occupied and retired coal-miners	0.7	1.0	1.1	1.5	2.0
Occupied and retired farm labourers	0.6	1.15	1.3	1.4	2.6

From this it is evident that the incidence of phthisis among coal-miners is definitely less than among farm labourers. On the other hand, Haldane points out that the death-rate from bronchitis in coal-miners is high when compared to other occupations. This, however he does not think bears any definite relation to the inhalation of coal dust, since the death-rate from bronchitis among coal-miners has markedly *decreased* in recent years although the amount of dust in the air of the mines has *increased* (largely owing to more powerful ventilation) within the same period.

Haldane is of opinion that the most probable cause of the diminution in the death-rate from bronchitis among coal-miners is to be found in the better ventilation of the mines and the consequent lessening of the respiratory movements. This latter would entail a smaller tendency to emphysema, and, following this, bronchitis.

Mavrogordato (1918) has made the most important contribution to the experimental study of dust-inhalation of recent years.

This author exposed guinea-pigs to different kinds of dust in a special chamber, the dusty atmosphere being maintained by a fan. His main conclusions are as follows:

Coal is rapidly eliminated from the lungs provided that the amount inhaled is moderate.

Flue dust causes plaque formation and patches of broncho-pneumonia.

Shale gives results intermediate between coal and flue dust: no permanent lesions occur, but the rate of elimination is slow as compared to coal.

Transvaal dust causes marked lung changes and remains (largely) in the lungs.

Pure precipitated silica is rapidly eliminated.

A *mixed dust*—i.e. flint and coal—was finally tested. The results of this were not conclusive, but Mavrogordato inclines to the view that the "lungs would practically free them-

seles from flue or crystalline silica dusts if these dusts enter in small quantities only with coal." The greatest length of time which elapsed in any of these experiments between the last exposure to dust and the autopsy was 10 months.

Haldane (1918) has dealt with the importance of dust-inhalation in various occupational diseases. He points out that the lesions produced by quartz dust, which are usually attributed to the sharpness of the particles, are probably not due at all to this, since microscopically such particles are neither sharper nor more angular than those of other and more innocuous dusts. Haldane also stresses the fact that the harmful dusts are those which are not eliminated with sufficient rapidity from the lungs, while the harmless ones are taken up by cells and quickly eliminated.

Sewell (1918) has made a careful study of the phagocytic powers of alveolar epithelium. His technique consisted in administering to rabbits preliminary intravenous injections of carmine; 24 hours after the last injection another—and this time intratracheal—injection was made. The substances introduced into the trachea included Indian ink in normal saline, pigeon's blood, *Staphylococcus pyogenes aureus* in normal saline and spores of *Oidium albicans* in normal saline. The animal was killed 3 to 24 hours after the intratracheal injection. These experiments showed that whereas the phagocytic cells within the alveoli, which Sewell seems largely to consider as being derived from the alveolar epithelium, took up the substance injected into the trachea, they failed to ingest the carmine particles. The latter, on the other hand, were engulfed by the macrophages of the blood. The great merit of Sewell's experiments is that they appear conclusively to demonstrate that the intra-alveolar macrophages or dust-cells are derived from alveolar epithelial cells and do not represent the macrophages of the blood stream.

Permar (1920, 1, 2, and 3), after repeating Sewell's experiments with a slightly modified technique, has come to the conclusion that the "Dust-cells" are endothelial elements derived from the blood capillaries of the alveoli. These cells, after irruption into the alveolar cavities and phagocytosis of pigment particles, slowly make their way back through the alveolar walls into the lymphatics. Stages in the proliferation of the endothelial elements—destined to become dust-cells—are figured by Permar. The fact that the dust-cells, though of vascular origin, are not to be found in the general circulation, is regarded by this author as evidence of their local origin and distribution.

Westhues (1922), after making experiments with a technique very similar to that of Permar, has come to conclusions diametrically opposed, in that Westhues regards the dust-cells as being derived from the alveolar epithelium and not from the endothelial cells of the pulmonary capillaries.

Gye and Kettle (1922, 1 and 2) have investigated the relation between silicosis and tuberculosis along novel lines. These authors found that the subcutaneous injection of mice with finely powdered silica (either insoluble or colloidal) in saline produces characteristic lesions at the site of inoculation. These changes comprise a focus of coagulative necrosis surrounded by leucocytes; this focus is subsequently absorbed and replaced by an inflammatory fibrosis. It was also noted that the injection—simultaneous or previous—with Tubercle Bacilli of such an area was followed by marked proliferation of the bacilli. On the other hand, subcutaneous injection of Tubercle Bacilli, without injection of silica, caused but slight tissue response with relatively slight multiplication of the Tubercle Bacilli, the mouse possessing a high degree of natural immunity towards these organisms. Clearly, then, the presence of silica in subcutaneous connective tissue favours the survival and multiplication of the Tubercle Bacilli. This the authors suggest is due to silica being a cell-poison, with the result that the bacilli thrive in the silica-laden areas of damaged and necrotic tissue. In the case of phthisis subsequent to pulmonary silicosis an additional factor is involved—the disorganisation of the lymphatics of the lung by the silica¹.

¹ On going to press (May, 1924), Fenn's admirable studies on the rate of phagocytosis of coal and quartz particles came to my notice. This observer found that carbon particles were ingested by leucocytes about four times as readily as quartz; quartz, in acid solutions, was ingested more rapidly than carbon, but carbon more readily than quartz in alkaline solutions. The applications of these quantitative studies of Fenn's to other dusts would be of interest. (Fenn, W. O., "Phagocytosis of Solid Particles," Studies Nos. I and III, *Jour. Gen. Physiol.* III. 1921, pp. 439 and 575.)

3. MATERIAL AND METHODS.

The animal used throughout these experiments was the guinea-pig. The dust cloud was raised in the apparatus already employed by Mavrogordato (1918) consisting of a large wooden box bearing a piece of plate-glass as a cover and a two-bladed fan working in a circular channel at its bottom. The animals were placed on a wire grid midway between the top and bottom of the box.

Various methods of killing the guinea-pigs were tried. Killing by chloroform or ether anaesthesia was unsatisfactory on account of the great congestion produced in all the viscera—and in the lungs in particular—just prior to death. The following method gave the most satisfactory results: One of the carotids was rapidly dissected out under chloroform anaesthesia; the artery was severed and the animal bled to death. To reduce collapse of the lungs to a minimum the trachea was ligatured before opening the thorax and removing the lungs. The latter were fixed in corrosive sublimate—formol, a mixture which was found to have the great advantage over Bouin's fluid—so commonly employed for fixing lungs—that red and white blood corpuscles are accurately fixed. The method of distension fixation was tried and not found to be as satisfactory as the technique of immersion fixation of lungs and heart after occlusion of the trachea. It is comparatively easy in one and the same specimen to burst the alveoli in some parts of the lung and not sufficiently to distend them in others.

The lungs were embedded in paraffin wax under reduced atmospheric pressure (450 mm. Hg). This method of embedding is almost essential for the successful penetration of the paraffin into the air-laden lungs. The only other alternative is to keep the pieces of lung for weeks in 70 per cent. alcohol until the air has disappeared, and then imbed at atmospheric pressure in the ordinary manner. One precaution, however, has to be observed when employing the technique of vacuum embedding. The reduction of the pressure has to be slowly made, for unless this is done, the alveoli are distended and may even burst, thereby producing an artefact microscopically indistinguishable from true pulmonary emphysema. The production of this artefact was guarded against by ascertaining the rate and degree of reduction of pressure which could safely be applied to normal guinea-pig lungs, the rate of removal of air from the pathological material being kept well within these limits.

The standard staining method was Ehrlich's haematoxylin and eosin. In cases of suspected fibrosis Heidenhain's iron haematoxylin counterstained with van Gieson's or Mallory's connective tissue stain were employed.

For the bacteriological examination of the sections the Claudius-Neutral Red and Ziehl-Neelsen stains were used as routine methods.

Many other stains were also employed from time to time, but no useful purpose would be served by describing them here.

4. THE HISTOLOGY OF THE NORMAL GUINEA-PIG LUNG.

To establish as far as possible the limits of normality, the lungs of animals which had never been placed in the dusting machine were examined at intervals. This precaution is essential in view of the fact that pigment particles can be found in the lungs of most—if not all—domesticated animals.

As the result of the microscopical examination of the lungs of seven control guinea-pigs, the following points were confirmed or established:

1. The lungs of guinea-pigs kept under the usual conditions of confinement contain fine black intra-cellular dust particles. Although there is no difficulty in finding these with the microscope, they are always relatively scanty when compared to the pigment particles seen in the lungs of the dusted animals. This dust is probably of the nature of fine carbon particles derived from the air, though the possibility of dust from the straw bedding, etc., cannot be excluded.

2. Fibrous (*i.e.* collagen) connective tissue is scanty in the guinea-pig lung as compared to its development in the larger mammals and especially man. In the normal guinea-pig lung fibrous tissue is only found in the following situations:

- (a) Beneath the pleura as a thin sub-serous layer.

- (b) Around the large and medium-sized bronchi.

- (c) Around the larger branches of the pulmonary veins. As a consequence of the paucity of fibrous tissue, the guinea-pig lung is of very delicate texture (see Plate VI, figs. 1 and 2).

3. Elastic tissue is highly developed around the alveoli of normal guinea-pig lung.

4. The presence of eosinophils, often in large numbers, in and around both epithelium and connective tissue of the bronchi and trachea is normal. This observation was first made by Opie (1904).

5. The media of the smaller branches of the pulmonary arteries, as noted by Jordan (1920), forms curious oval thickenings in the wall of these vessels.

6. The lungs of control animals often show small areas of congestion, as already pointed out by Beattie (1912). Examination of such areas reveals congestion of the smaller blood-vessels, capillary engorgement, and (usually) an increase in the number of alveolar epithelial cells. The alveolar walls are consequently somewhat thickened. Such an area of congestion is shown in Plate VI, fig. 3.

7. Small areas of broncho-pneumonia were noted in the lungs of three out of the seven control animals. No symptoms were noticeable in these guinea-pigs. Examination of sections stained by the Ziehl-Neelsen and Claudius-Neutral Red methods failed to show any organisms, although the presence of broncho-pneumonic foci is strongly suggestive of an infection.

8. The bronchial glands of normal guinea-pigs sometimes show scanty black intra-cellular particles. Eosinophil infiltration of both gland substance and capsule is not uncommon, as noted by Opie (1904). Occasionally eosinophils may be found in the afferent lymphatics of the gland.

This infiltration of lymphoid, peri-bronchial and peri-vascular connective tissue is to be regarded as perfectly normal in guinea-pigs.

Lymphoid tissue is widely distributed throughout the normal guinea-pig lung. Not only are small nodules present in the peri-bronchial connective tissue, but they also occur in the lung parenchyma itself.

To ascertain whether the lesions observed in the dusted animals were due solely to the dust and not to the draught created within the machine by the revolving fan, the following control experiment was devised:

Four animals were exposed to the draught within the machine for the same period as the dusted animals—*i.e.* two hours daily for fourteen days (Sundays excluded).

Three of the guinea-pigs were perfectly healthy at the end of the experiment, one coughed, panted, and was very weak from the tenth day of exposure onwards.

Microscopic examination of the lungs of these animals was made 24 hours after the last exposure.

The lungs of the three healthy animals show large areas of normal lung. Interspersed among these, however, are patches of slightly thickened alveoli, and, in each of the specimens, a small area of broncho-pneumonia.

The lungs of the diseased guinea-pig show a massive broncho-pneumonia, with marked "cuffing" of the pulmonary veins by lymphocytes. The examination of sections stained for bacteria by the Ziehl-Neelsen and Claudius-Neutral Red methods failed to reveal organisms.

Lung changes such as those found in the three healthy animals exposed to the draught are so frequent in guinea-pigs kept in confinement that it would be unwarranted to ascribe them to the draught in the machine, which, moreover, is very slight. The massive broncho-pneumonia of the fourth animal was probably due to infection, since guinea-pigs from the normal stock have died from time to time and presented similar lesions.

5. THE LESIONS CAUSED BY THE DUSTS USED IN THESE EXPERIMENTS.

The mode of dusting was as follows: The animals were exposed to the dust for two hours daily over a period of 14 days, Sundays excluded. Hence the total number of days on which the guinea-pigs were exposed was 12. The dusts were measured by volume, and the total amount of dust placed in the machine for each period of two hours was 135 c.c.

(1) INHALATION OF A MIXTURE OF FLINT AND COAL.

Series 1.

Two parts of flint to one of coal by measure, the total volume of dust administered at each dusting period being 135 c.c. The lungs of most of the animals of this series showed, at autopsy, some degree of pigmentation.

No. 1. Animal killed three hours after the last exposure.

Microscopically there is thickening and cell proliferation of the alveolar epithelium. The lungs contain much intra-cellular dust, some of the dust-cells being free within the alveolar cavities while others are still attached to the alveolar walls. The smaller bronchi contain plugs of dust-cells. The bronchial glands show no more dust than is usually found in control animals (see p. 444).

No. 2. 24 hours after exposure.

Microscopically the alveoli show the same changes as in the 3-hour specimen; in addition, there are areas of collapse and, rarely, of oedema. Dust particles numerous and evenly distributed as shown in Plate VI, fig. 5. Bronchi often plugged. Bronchial glands normal.

No. 3. One month after exposure.

Large areas of lung are normal except for the presence of a little dust. Elsewhere there is capillary congestion, proliferation and thickening of the alveolar epithelium. Dust-cells are abundant and there is marked eosinophil infiltration of the lung substance. Smaller bronchi sometimes plugged. Some dust in the peri-bronchial lymphatics. Bronchial glands contain more dust than is normal for control guinea-pigs.

No. 4. Six months after exposure.

Areas of normal lung alternate with areas of cell-proliferation and capillary congestion. More normal tissue in the lobes than in the apices. No dust in the lymphoid tissue of the lung. There is less dust than in No. 3. There are small areas of incipient *plaque-formation*.

By *plaque-formation* is meant the appearance of conglomerations of thin squame-like cells, the cytoplasm of which is packed with dust particles. The nuclei of such cells are often multiple and degenerate. Vascular connections

are lacking within these areas of plaques, which I interpret as an index of incomplete phagocytic response on the part of the dust cells: the dust is ingested, but the cells degenerate and die before they have removed it from the lung substance. Areas of plaque-formation, if of any size, represent permanently damaged areas of lung substance. I have noticed, however, that while some of the lungs of animals exposed to a given dust contain plaques, the lungs of animals from the same series, but killed several months later, may not show them. Often this can only be ascribed to differences in the susceptibility of the lungs in different animals. But not always, and in such cases I think that the disappearance of the plaques from the lung is due to plaque-formation having occurred *not* in the lung parenchyma but inside the lymphatics of the lung—notably the sub-pleural lymphatics. Under such conditions, the necrotic dust-containing cells are somewhat isolated from the lung parenchyma and fibrosis does not supervene. On the other hand, disintegration of the plaques within the lymphatics may well lead to the removal of the dust to adjacent lymphoid tissue, and notably the bronchial glands. It is significant that some of the smaller plaques appear to be encircled not by proliferated alveolar epithelium but by flattened cells strongly suggestive of the endothelium of a lymphatic. Large plaques, however, I regard as permanent lesions in contact with the smaller—intra-lymphatic—plaques. Plaques are figured in Plate VII, figs. 13 and 14.

No. 5. 9½ months after exposure.

The alveolar epithelium shows extensive thickening and cell-proliferation. There are areas of broncho-pneumonia as shown in Plate VI, fig. 6. Eosinophil infiltration is very slight. Dust-cells—often multi-nucleate—are present in fair numbers. The bronchial glands contain a little intra-cellular dust. The changes are more pronounced in the apices than in the lobes.

No. 6. 12½ months after exposure.

Large areas of normal or slightly emphysematous lung alternate with small patches of broncho-pneumonia, which latter contain dust-laden plaques. Plaque-formation is especially pronounced beneath the pleura. Changes less marked in lobes than in apices. Plaque-formation in these lungs is shown in Plate VII, figs. 13 and 14.

No. 7. 16 months after exposure.

Large areas of lung approximate towards the normal except for the presence of dust-cells in the alveolar walls. In some parts of the lung broncho-pneumonia is present. In these regions (see Plate VII, figs. 8 and 9) the alveolar walls are thickened and the alveolar cavities largely obliterated. There are masses of dust-containing cells aggregated into small plaques. "Cuffing" by lymphocytes of some of the smaller branches of the pulmonary veins has occurred. No fibrosis; no eosinophil infiltration. The lymphoid tissue of the lung contains a little dust, the bronchial glands a good deal. The most damaged areas of the lung are those containing the most dust, but there is considerably less dust in the specimen than in No. 6 of this series.

Series 2.

A few animals were exposed to the same amount of flint and coal for the same dusting periods and for the same number of days as in Series 1, but the

relative proportions of the two dusts were reversed—*i.e.* 2 parts of coal were mixed with 1 part of flint by measure (instead of 1 of coal to 2 of flint).

The records of this series may be summarised as follows:

Two animals, *killed 11 days after exposure*, show general thickening and proliferation of the alveolar epithelium, patchy areas of broncho-pneumonia and much dust.

A specimen of lung *3½ months after exposure* shows marked broncho-pneumonia with massive eosinophil infiltration. The lesions are most pronounced in the lobes—an exceptional occurrence. There is a fair amount of intra-cellular dust.

The lungs of two animals *killed 9 months after exposure* reveal a fair amount of normal tissue, areas of thickened alveolar epithelium and, specially in one of the specimens, patches of broncho-pneumonia without eosinophil infiltration. The bronchiolitis has considerably subsided. Giant cells containing dust particles are present. No fibrosis; no plaque-formation.

(2) INHALATION OF CHINA CLAY.

(AS SUPPLIED TO THE POTTERIES.)

Series I.

The standard amount of dust (135 c.c.) was administered at each dusting period. At autopsy the lungs showed greyish streaks on their surfaces.

No. 1. Animal killed 24 hours after the last exposure.

The alveolar walls are somewhat thickened—especially in the apices. Large areas of lung are normal except for the presence of inspissated particles—dark grey to brown—plastered here and there on to the alveolar walls. Only a little of the dust is intra-cellular. The bronchi contain a few dust-cells and a little free dust. The bronchial glands are normal.

No. 2. One month after the last exposure.

The condition of the lungs and bronchial glands is as for No. 1 of the series with the following differences: There is slight collapse beneath the pleura in some parts of the lung (see Plate VII, fig. 10), while the dust particles are now nearly black.

No. 3. Two months after the last exposure.

In some parts the alveolar walls are thickened and there is capillary congestion. Large areas of lung are normal except for the presence of a moderate amount of intra-cellular dust. The dust-cells are nearly always attached to the alveolar walls.

No. 4. Three months after exposure.

The lesions have progressed. There are numerous areas showing great proliferation and thickening of the alveolar epithelium. Capillary engorgement is marked. Dust-cells, both free and attached, are abundant. There is capillary bronchitis in many areas of the lung, but dust particles are scanty among the cell-débris in the lumina of the bronchi.

No. 5. Four months after exposure.

The lung changes are as for No. 4 of the series while, in addition, incipient plaque-formation has set in. The lungs still contain a moderate amount of dust, a little of which can be seen within the macrophages in the lymph sinuses of the bronchial glands.

No. 6. Six months after exposure.

Thickening of the alveolar walls is general, except where areas of emphysema occur. There is capillary congestion and eosinophil infiltration. There are areas of broncho-pneumonia. Such an area is depicted in Plate VII, fig. 11; in it can be seen plaques and a massive eosinophil infiltration. Dust is present in moderate amount. The lesions are more severe in the apices than in the lobes.

Series 2.

The experiments were repeated, using the same dust, but at half the concentration previously employed—*i.e.* 65 c.c. of china clay being administered at each dusting period.

The lesions caused show no appreciable difference when compared with Series 1. The last animal of Series 2, killed nine months after exposure, contained many plaques in its lungs. The bronchial glands showed some intra-cellular dust.

Series 3.

This series was dusted as follows:

1 exposure per week for 14 days,

2 exposures per week for 14 days,

1 exposure every 2 days for 16 days.

The amount of dust administered at each dusting period was as for Series 2 (*i.e.* 65 c.c.).

It will be seen that 14 exposures (two more than in the other series of experiments) were given over a period of about 6 weeks (44 days) instead of 14 days. Further, the intervals between the exposures were progressively shortened as the experiment proceeded.

Animals examined 2½ and 7 months after the last exposure showed not only lesions less severe than in Series 2, but also evidence of a far more active phagocytosis of the dust particles, which were found in both bronchial and abdominal lymph glands. The last two animals examined, 7 months after exposure, showed neither plaque-formation nor fibrosis.

(3) INHALATION OF FELSPAR.

Standard exposures and concentration of the dust. At autopsy the lungs of the animals of this series were normal in appearance up to three months after exposure. Later specimens showed black spots scattered over the surface of the lungs, and, sometimes, areas of congestion.

No. 1. Animal killed six hours after the last exposure.

Microscopically there are large areas of normal lung except for the presence of dark brown dust particles plastered on to the alveolar walls. In some places, and especially beneath the pleura (see Plate VII, fig. 12), the alveolar epithelium is proliferating and thickened. The amount of dust is moderate, its distribution patchy. The bronchial glands are normal.

No. 2. One month after exposure.

The lung changes are as in No. 1 of this series. No free dust cells were noted. There is some bronchitis. The bronchial glands are normal.

No. 3. Two months after exposure.

There are large areas of proliferating and congested alveolar epithelium. Dust-cells mostly attached to the alveolar walls, though a few are present in the smaller bronchi. Small plaques are present (see Plate VII, fig. 14) and the general lesion is that of a bronchopneumonia with eosinophil infiltration. The bronchial glands contain a little intra-cellular dust.

No. 4. Three months after exposure.

Lesions as for No. 3, but the plaque-formation and the bronchitis have increased. There is less dust within the lungs than in No. 2 of the series (killed one month after exposure) and the particles are darker.

No. 5. Five months after exposure.

General thickening of the alveolar epithelium. There are areas of emphysema and also abundant plaques. Some diapedesis of the red blood corpuscles. The bronchial glands are free from dust.

No. 6. Nine months after exposure.

The extensive changes still persist, but are more intense in the lobes than in the apices. No dust—either free of intra-cellular—within the bronchi. The bronchial glands contain a fair amount of intra-cellular dust.

No. 7. Twelve months after exposure.

The lesions are less marked than in No. 6, large areas of lung showing only moderate thickening of the alveolar epithelium (see Plate VII, fig. 13). The eosinophil infiltration has subsided. There is still much dust in the lungs. There is a mild fibrosis, the collagen fibres being derived from the peri-bronchial and peri-vascular connective tissue.

(4) INHALATION OF GROUND PITCHER.

(AS USED IN THE POTTERIES. GROUND PITCHER DENOTES THE
CRUSHED-UP EARTHENWARE AFTER FIRING.)

Standard exposures and concentration of the dust. At autopsy the lungs were always pigmented.

No. 1. Animal killed 24 hours after the last exposure.

Microscopically the lungs show a general thickening and proliferation of the alveolar walls. In one apex there is an area of broncho-pneumonia with eosinophil infiltration as depicted in Plate VIII, fig. 15. There is much dust, the particles varying from yellow to black. Some of the dust is intra-cellular; the dust-cells are only rarely free. Bronchitis is present, but the bronchi are dust-free. The bronchial glands are normal.

No. 2. Three months after exposure.

Lesions as in No. 1 of the series; in addition, plaque-formation has set in and many of the macrophages of the bronchial glands contain dust particles.

No. 3. Four months after exposure.

The broncho-pneumonia has been largely replaced by interstitial changes, which, where pronounced, comprise an invasion of the dust-laden and greatly thickened alveoli by strands of fine fibrous tissue (see Plate VIII, fig. 16). The peri-vascular lymphatics often show accumulations of lymphocytes around and within them. Catarrhal changes are present in the bronchi. The bronchial glands contain intra-cellular dust.

No. 4. Six months after exposure.

The lungs show general interstitial changes and also large patches of broncho-pneumonia (see Plate VIII, fig. 17). In some of the areas of plaque-formation many young collagen fibres and fibroblasts are apparent. There are still many black dust particles in the lungs. The bronchial glands contain dust-laden macrophages, apparently blocking the lymph sinuses, in large numbers as shown in Plate VIII, fig. 18.

No. 5. Nine months after exposure.

This animal has a large abscess containing cheesy pus on the left side of the head affecting the eye on the same side. The lungs show extensive broncho-pneumonia, some areas of emphysema, and a little normal tissue (see Plate VIII, fig. 19). The bronchial glands contain much dust, the lymph sinuses being blocked by conglomerations of macrophages containing black pigment particles.

(5) INHALATION OF PURE AMORPHOUS SILICA.

(DEHYDRATED AND RENDERED INSOLUBLE BY HEATING.)

Usual concentration and exposures. At autopsy the lungs of the animals of this series showed grey to black pigmentation, and, sometimes, punctiform haemorrhages.

No. 1. Animal killed a few hours after the last exposure.

Microscopically there is general thickening of the alveolar walls, the dust-cells (often multi-nucleate) being attached to them. There is general capillary congestion, and, occasionally, haemorrhage into the alveoli. Some eosinophil infiltration. The bronchi are catarrhal. Much dust is present. The greater severity of the lesions in the apex as compared to the lobe is shown in Plate IX, fig. 25.

No. 2. One month after exposure.

The changes are as in No. 1 of the series, but plaque-formation has also appeared. Eosinophil infiltration is very slight. The bronchial glands contain intra-cellular dust particles.

No. 3. Four months after exposure.

Bronchiolitis and a patchy broncho-pneumonia persist—the latter associated with eosinophil infiltration. Plaques are absent. Bronchial glands normal.

No. 4. 5½ months after exposure.

There are large areas of slightly thickened alveoli. In the azygos lobe, however, consolidation is complete. Here the capillaries are engorged and beaded, the alveolar epithelium is absent, and a cellular exudate fills the alveoli. There is much dark brown intra-cellular dust. The peri-vascular connective tissue shows slight hypertrophy. The condition in this azygos lobe is such as to suggest a chronic broncho-pneumonia associated with a slight fibrosis.

No. 5. Eight months after exposure.

The bronchiolitis and broncho-pneumonia persist. Elsewhere the alveolar walls are thickened. The most damaged areas show a slight fibrosis. The lungs contain a fair amount of dark brown dust, but there is no plaque-formation.

No. 6. 13 months after exposure.

The changes are much as in No. 5 of the series, but there is no fibrous tissue increase. In addition, sub-pleural plaques are in evidence.

No. 7. 23 months after exposure.

The lesions in this specimen comprise extensive bronchiolitis, large areas of broncho-pneumonia with eosinophil infiltration, patches of emphysema and sub-pleural plaque-formation. Much dust still remains in the lungs. The bronchial glands contain abundant dust particles.

(6) INHALATION OF PURE FLINT.

(AS USED IN THE POTTERIES.)

Dusting was carried out according to the usual method. At autopsy the lungs showed a streaky pigmentation and, usually, scattered haemorrhagic areas.

No. 1. Animal killed 22 hours after the last exposure.

The lungs are histologically normal over large areas; elsewhere, and especially in the

apices, there is acute capillary congestion, some haemorrhage into the alveolar cavities, and proliferation of the alveolar epithelium. Attached to the latter are many dust-cells which are only occasionally found free within the alveoli. There is some bronchiolitis. The most damaged areas show slight eosinophil infiltration. (See Pl. IX, fig. 26.)

No. 2. One month after exposure.

The lesions are as in No. 1 only more pronounced. There is also a patchy broncho-pneumonia and incipient plaque-formation. The bronchial glands contain some intracellular dust.

No. 3. Four months after exposure.

The condition of the lungs in this specimen is as in No. 2 of the series, except that dust-containing giant cells have appeared, also areas of emphysematous tissue. The bronchial glands are normal.

No. 4. Seven months after exposure.

The patchy condition of the lungs persists. The areas of broncho-pneumonia show no eosinophil infiltration. There are large necrotic dust-containing cells in some parts of the lung; these probably represent very early plaque-formation. Giant cells, containing dark brown dust particles, are abundant.

No. 5. Nine months after exposure.

The lesions are as in No. 4 of the series. There is, however, some eosinophil infiltration of the areas of broncho-pneumonia.

Nos. 6 and 7. 12½ and 13 months after exposure.

The lung changes are very similar in both these specimens, which show large areas of broncho-pneumonia heavily infiltrated with eosinophils. Pigment-containing giant cells are common. There are no plaques, neither is there any evidence of an increase in the fibrous tissue. Much dust still remains in the lungs. The lesions are far more marked in the apices than in the lobes, as can be seen by comparing Figs. 27 and 28 (Pl. IX). The bronchial glands contain a moderate amount of dust.

(7) INHALATION OF COAL DUST.

A. As a preliminary experiment to test the effect of small quantities of coal dust on the lungs, a guinea-pig was exposed to the usual concentration of dust for two days only. It was killed immediately after exposure.

Histologically most of the pulmonary alveoli are normal, the chief changes being a mild catarrhal reaction of the bronchi (see Plate X, fig. 29). The latter contain plugs of cells (chiefly eosinophils) and a good deal of dust (mostly free).

B. Animals were exposed to coal dust under the standard conditions—*i.e.* two hours' exposure daily for 14 days. At autopsy the lungs showed dark streaks over their surfaces, and, occasionally, a few small haemorrhagic areas. The lungs of the last specimen examined (19 months after exposure) were macroscopically normal.

No. 1. Animal killed six hours after the last exposure.

There is a general thickening of the alveolar epithelium, and also some areas of broncho-pneumonia as shown in Plate X, fig. 30. Dust is abundant, both in the alveoli and the bronchi, but it is in process of active phagocytosis by the dust-cells. Bronchitis is fairly general. The bronchial glands contain a moderate amount of dust.

No. 2. One month after exposure.

Large areas of lung substance are only slightly thickened; elsewhere there are patches of broncho-pneumonia, heavily laden with dust, and infiltrated with eosinophils. One of the lobes is emphysematous. Bronchial catarrh is marked, the plugs of cells within the bronchi containing a little free coal dust. The bronchial glands contain a moderate amount of dust.

No. 3. Four months after exposure.

The lung substance in the lobes is almost normal, but the apices show some areas of broncho-pneumonia. There is less dust present in the lungs of this specimen than in No. 2 of the series. The smaller bronchi are catarrhal and contain a few dust-cells. The lymphoid tissue of the lung contains some dust.

No. 4. Two animals nine months after exposure.

These specimens show a general though slight proliferation of the alveolar epithelium associated with occasional sub-pleural patches of broncho-pneumonia. Dust-cells abound; sometimes they are free within the alveolar cavities, though more usually they project into these from their points of attachment to the alveolar walls. The lymphoid nodules in the lung substance contain a little dust. In one of the specimens there are dust particles within the peri-vascular lymphatics, as well as in the endothelial cells of the intima, of branches of the pulmonary vein. The bronchial glands, only examined in one of the animals, contained much dust in the lymph sinuses.

No. 5. 19 months after exposure.

There is a good deal of slightly emphysematous but nearly dust-free lung. Some areas of broncho-pneumonia. Many of the bronchi are catarrhal, but do not contain any dust. A portion of one of the most damaged areas in an apex is shown in Plate X, fig. 31. The bronchial glands contain a moderate amount of dust. Neither plaque-formation nor fibrosis were noted in this animal or in any others of the series.

(8) INHALATION OF SHALE DUST.

(AS USED FOR DUSTING IN COAL-MINES.)

The standard exposures were employed in this series. At autopsy the lungs showed slight pigmentation and an occasional small haemorrhagic area.

No. 1. Animal killed immediately after the last exposure.

There are large areas of normal or slightly thickened lung substance. Black intra-cellular dust is present in moderate amount, the dust-cells usually projecting into the alveolar cavities from the point of attachment to the epithelium. Some dust-cells are also present in the bronchi, these latter being catarrhal. There are some small areas of broncho-pneumonia.

No. 2. One month after exposure.

General proliferation and thickening of the alveolar epithelium has set in. There are areas of sub-pleural broncho-pneumonia as evidenced by Plate X, fig. 32. Eosinophil infiltration and capillary engorgement are also present. There is some bronchitis. The nodules of lymphoid tissue adjacent to the larger blood-vessels contain a little dust.

No. 3. Two months after exposure.

The lesions are of the same type as in No. 2 of the series but more pronounced. The bronchial glands contain dust.

No. 4. Three months after exposure.

The changes are less marked than in No. 3 of the series. The amount of dust has also considerably diminished. Emphysema is present in some areas as shown in Plate X, fig. 33.

Nos. 5 and 6. Two animals—8 and 8½ months after exposure.

There is slight but fairly general thickening of the alveolar epithelium in both these specimens. In one (No. 6) there are a few small areas of broncho-pneumonia (see Plate X, fig. 34) and isolated patches of plaque-formation. There are, however, no gross lesions and the amount of dust in the lungs has undergone considerable reduction. Some of the bronchi are catarrhal. The peri-bronchial lymphoid tissue in the lung contains a little dust in one of the animals (No. 5), while the bronchial glands—only examined in No. 6—contain a fair amount of dust. There is no fibrous tissue increase.

(9) INHALATION OF IGNITED SHALE.

The shale used in these experiments was heated to redness in the electric furnace so as to destroy all organic matter.

Standard concentration and exposures. At autopsy pigmentation of the lungs, chiefly apical, was present.

No. 1. Two animals killed 24 hours after the last exposure.

In both specimens proliferation and thickening of the alveolar epithelium is general. There are extensive areas of broncho-pneumonia with pseudo-eosinophil infiltration and some "cuffing" of the blood-vessels by lymphocytes. Intra-cellular dust is abundant. The bronchi contain dust and are catarrhal.

No. 2. Two animals six weeks after exposure.

Changes slightly less marked than in No. 1. The amount of dust in the lung substance is much the same, but intra-bronchial dust particles are very scanty. The bronchial glands contain much intra-cellular dust.

No. 3. Two animals four months after exposure.

There are fair-sized areas of normal lung alternating with patches of broncho-pneumonia. Dust is still present in considerable amount. A few isolated areas of plaque-formation. The bronchial glands contain abundant intra-cellular dust particles.

No. 4. One animal six months after exposure.

The condition of the lungs is much as in No. 3. Intra-cellular dust is present in fair amount. Bronchi catarrhal and dust-containing.

No. 5. One animal eleven months after exposure.

A few areas of consolidation. Large areas of normal or nearly normal lung substance. A few small areas of plaque-formation. The dust is considerably reduced in amount. There is no fibrosis. One of the areas of consolidated tissue contains giant cells within which are granular oxyphil bodies. These, while certainly not bacterial, are of uncertain origin. Probably they represent the granules of eosinophil cells previously phagocytosed.

(10) INHALATION OF DRIED EARTH.

Ordinary garden earth passed through a sieve in order to eliminate the coarser particles.

Usual concentration and exposures.

At autopsy the lungs showed marked pigmentation in No. 1, very slight pigmentation in the succeeding animals.

No. 1. Two animals killed three days after last exposure.

In both specimens there is general thickening of the alveolar epithelium, some areas of

haemorrhage into the alveoli, and numerous intra-cellular particles. There is slight bronchial catarrh, and, in one of the specimens, a few broncho-pneumonic patches.

No. 2. Two animals killed two months after exposure.

The lesions have slightly regressed. Much dust is still present both in the lungs and in the bronchial glands.

No. 3. One animal killed seven months after exposure.

Areas of proliferated alveolar epithelium and broncho-pneumonia (with eosinophil infiltration) are still to be found. There is slight bronchitis but no intra-bronchial dust. The lung substance contains a fair amount of dust. No plaque-formation.

No. 4. Two animals nine months after exposure.

There is widespread thickening of the alveolar epithelium. A few small areas of broncho-pneumonia. The lungs contain a fair amount of intra-cellular dust while the bronchi are dust-free. Neither plaque-formation nor fibrosis. The lungs are microscopically negative for organisms.

(11) INHALATION OF IGNITED EARTH.

Sifted garden earth heated to redness in the electric furnace.

Standard concentration and exposures.

Only three animals have been examined owing to an epidemic of pneumonia which killed all the other animals in this, and also large numbers in the other, groups.

No. 1. Two animals killed 24 hours after the last exposure.

In one of the animals there is intense pulmonary congestion—undoubtedly agonal. Apart from this the lesions are similar in both specimens, and comprise small areas of thickened alveolar epithelium. Intra-cellular dust is plentiful. There is no bronchitis but much intra-bronchial dust.

No. 2. One animal killed 3½ months after exposure.

There are areas of proliferated alveolar epithelium and plaque-formation (the latter very scanty). Some bronchitis. Over the lobes there is an extensive, old, organised pleurisy with some fibrotic ingrowth into the underlying lung substance. Both lungs and pleura are microscopically negative for bacteria. The bronchial glands contain a fair amount of intra-cellular dust.

(12) THE BACTERIOLOGICAL EXAMINATION OF THE SECTIONS.

The results furnished by staining sections of the lungs of those animals which showed pronounced lesions are summarised and briefly commented on below.

The Ziehl-Neelsen and Claudius stains were used as standard methods for each group of animals. Cresylecht Violet and Carbol-Thionin were also used in some instances. Since these two stains never revealed organisms not demonstrated by the standard methods, no reference is made to them in the following summary.

Summary of bacteriological examination of the lungs of Control and dusted animals.

Controls. Lungs and bronchi (medium and small) negative for micro-organisms in the three animals examined.

1. *Flint and coal (Series 1)*. A few gram-positive Streptococci and Diplococci in the bronchi. Lung substance negative.
2. *China clay (Series 1 and 2)*. Lungs and bronchi negative.
3. *Felspar*. Negative.
4. *Ground pitcher*. A few gram-positive Streptococci were once noted within a plug in a bronchus.
5. *Precipitated silica*. Negative.
6. *Flint*. Negative.
7. *Coal*. A gram-positive Streptococcus present in small numbers.
8. *Shale*. Negative.

Neither tubercle bacilli, nor, indeed, any lesions even remotely suggestive of pulmonary tuberculosis, were ever noted.

As shown by these findings, the presence of bacteria, even in the areas of broncho-pneumonia, was very rare, and, when present, they were always very scanty. The lungs of the control animals, including one broncho-pneumonic specimen, were negative. So also were the lungs of all the other groups with the exception of three.

The presence of bacteria in cases of human pneumoconiosis is, of course, well known. Thus, the expectoration of coal-miners "is found teeming with Staphylococci and many putrefactive organisms" (Summons (1907)). The fact that the broncho-pneumonic lesions were not found to contain organisms after treatment with bacterial stains should not be regarded as proof that such lesions neither contain organisms nor that they possibly represent, in part, the tissue-response to an infection. The microscopic examination of the consolidated portions suggests a chronic condition, primarily induced by the dust particles. It is hence possible that micro-organisms might have secondarily infected such areas in small numbers. And in such cases, only cultural methods—which are outside the scope of these observations—would be of use in detecting them.

6. DISCUSSION OF THE RESULTS.

Below are given the conclusions derived from the study of the lesions caused by the dusts used in these experiments.

At the outset I would stress the fact that such deductions as can be made from the study of the response of guinea-pig lungs to dust particles do not admit of a wholesale application to human beings.

It is essential clearly to recognise the limitations of observations such as these.

Firstly, the pulmonary response of the guinea-pig is often different in degree from that of man. Thus, fibrosis, the outstanding lesion directly due to the inhalation of flint dust in man, was never produced by intake of pure flint into the lungs of guinea-pigs in these experiments. This, I think, is due to an important histological difference in the two types of lung: that of man contains much fibrous tissue, that of small rodents very little.

Secondly, the nasal filter of the guinea-pig—from such examination as I have made of it after exposure to dust—appears to be greatly superior to that

of man. This means, of course, that the actual dust content of the inspired air would be less in the guinea-pig than in man.

Thirdly, an important difference between experimental and human pneumoconiosis arises from the fact that it is necessary, for practical reasons, when working with animals, to restrict the exposures to as short a period as possible—two hours daily for 12 days in the case of most of these experiments. A man, employed in a dusty profession, inhales dust for many years. Whence the necessity for using very high concentrations of dust in the case of animal experiments in order to obtain definite lesions after so short a series of exposures.

All this indicates the necessity for caution in the interpretation of animal experiments in terms of human pneumoconiosis. A dust which causes pulmonary damage in guinea-pigs but not, so far as is known, in man (*e.g.* china clay) can only be regarded as potentially dangerous—*i.e.* harmful if breathed at high concentrations—for human beings in the absence of other evidence.

(1) INHALATION OF A MIXTURE OF COAL AND FLINT.

It is well known that coal dust can be breathed in considerable concentration over prolonged periods without causing any damage to the tissues. It is equally well established that flint dust, even in the infinitesimal concentration in the flint-knapping industry, is extremely harmful. These experiments involving the inhalation of a mixed dust were performed so as to test Mavrogordato's hypothesis that a dust which stimulates phagocytosis—*e.g.* coal—if mixed with an inert dust—*e.g.* flint—will induce phagocytosis, and consequent elimination, of the flint particles as well as the coal.

This group of experiments contained the two following series:

Series 1. Coal and flint mixed in the proportion of one of coal to two of flint by measure (*i.e.* 25 per cent. and 75 per cent. by weight).

Series 2. Two parts of coal to one of flint by measure—*i.e.* the proportions of the dusts were reversed.

Analysis of Series 1 and 2. The immediate effects consist in proliferation of the alveolar epithelium, capillary engorgement and bronchitis. A patchy broncho-pneumonia with eosinophil infiltration appears after the first month. The amount of dust in the lungs undergoes diminution from the first to the sixth month. After 9½ months there is a change towards the normal; large areas of lung clear up though some extensive patches of broncho-pneumonia were still present in the latest specimen examined (16 months). Dust particles are usually present in the bronchial glands from one month after exposure onwards. The chief difference between Series 1 and 2 is that the lungs of the animals of the latter series never contained plaques. No fibrosis was noted in any of the specimens examined.

The histological evidence indicates that while coal and flint produce a brisk phagocytic response, the small amount of flint remaining behind in the lungs was sufficient, particularly in Series 1, to leave lasting though not extensive damage. But there is little doubt that the early phagocytic response initiated by the coal is responsible for the rapid elimination of much of the flint.

It is therefore conceivable that the dusting of gold-mines with coal dust might aid in the elimination of the very harmful particles of quartzite. According to the monograph of Silicosis of Watt, Irvine, Steuart and Johnson (1916), in the case of "machine miners who had worked $4\frac{1}{2}$ years underground, over 50 per cent. were found to be affected (including 'borderland' cases) with Silicosis. After $8\frac{1}{2}$ years the incidence of Silicosis is over 75 per cent. while after $15\frac{1}{2}$ years practically none of the men engaged in drilling operations are free from Silicosis." Furthermore, Silicosis is nearly always complicated by pulmonary tuberculosis (certainly in its later stages)¹.

These observations on guinea-pigs indicate that Haldane's (1918) proposal to dust gold-mines with coal dust might well be beneficial to the health of the miners. Possibly, too, coal dusting might be employed in some of the other trades which involve the inhalation of flint. And, finally, it is conceivable that pulmonary tuberculosis might favourably respond to a carefully graded inhalation of coal or soot particles.

(2) INHALATION OF CHINA CLAY.

China clay—also known as kaolin—is a fine white powder, used in the manufacture of earthenware and china. Chemically it is a silicate of alumina and "has very nearly the empirical composition $\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2 \cdot 2\text{H}_2\text{O}$ " (Mellor, 1914).

Three series of experiments were undertaken with this dust: the standard quantity of dust (135 c.c.) was put into the dusting machine for each period of exposure in Series 1. In Series 2 half the quantity was employed. In Series 3, while both the total amount, and each dose, of dust were the same as in Series 2, the dusting sessions were spaced out over a considerable period (over six weeks, instead of 14 days) as shown on p. 448.

Analysis of Series 1 and 2. The behaviour of the guinea-pig lung in the first two series towards this dust is interesting. Until three months after exposure the pulmonary reaction is very slight, being limited to mild proliferation of the alveolar epithelium. From three months onwards, a patchy broncho-pneumonia, with massive eosinophil infiltration, appears. The last specimen from Series 1, killed six months after exposure, showed plaque-formation and a pretty general bronchitis in addition to the above-mentioned changes.

In Series 2 exactly similar lesions were noted—even in the latest animal, examined nine months after exposure.

The bronchial glands showed some dust several months after exposure.

In Series 3 it was thought that the spacing out of the exposures would facilitate the removal of the dust, and that the phagocytic cells of the lung might thus be "trained" to deal with china clay.

The microscopic findings confirmed this supposition. The lesions were less marked than in Series 2, while a far more active phagocytosis was observed. Dust particles were removed to the bronchial, and even the abdominal, lymphatic glands.

¹ In a memoir which has recently come to my notice, Mavrogordato reports on some further experiments with coal and silica. He exposed animals to inhalations (a) of silica preceded by coal, (b) of silica mixed with coal, (c) of silica, followed by coal, and (d) of silica alone. His results indicate that "once Silica is fixed in the lung tissues, Coal exerts no eliminative effect,...but that a prior or even simultaneous exposure to coal dust appears to set up a condition in the lung which is inimical to the fixation of silica" [Mavrogordato, "Studies in Experimental Silicosis" (1922)].

From the above it can be seen that there is a long latent period—at least one month—during which phagocytic response of the lungs is almost absent. In fact, the dust particles can be seen lying against the alveolar epithelial cells which only very rarely, at this stage, show any signs of engulfing them. From three months onwards phagocytosis occurs, but the cells seem to die soon after they have taken up the dust. Consequently, much remains in the lungs, thereby initiating plaque-formation and broncho-pneumonia.

The great degree of harmfulness of china clay towards the guinea-pig lung is shown by the fact that even when inhaled at half the standard concentration the lesions produced are quite as severe as when inhaled in the standard quantity. By spacing out the doses, however, it seems possible fairly efficiently to train the lungs to deal with this dust. This is in striking contrast with the effects produced by daily inhalations of even smaller quantities of dust.

Curiously enough, there does not seem to be any evidence that china clay is particularly harmful to the lungs of human beings.

When this substance is used in the manufacture of china and earthenware, Dr White, of the Royal Worcester Porcelain Works, tells me that it is not breathed by the workers; the clay, as received at the works, contains about 10 per cent. of water and therefore does not fly. Further, the mixing is done wet. These facts I was able to note for myself when visiting the above establishment.

Although industrial medicine furnishes no definite evidence that the inhalation of china clay is productive of pulmonary lesions in man, I feel that these experiments indicate that china clay is perhaps a potentially dangerous dust. That is to say, that it might well be harmful to man if breathed in higher concentrations than those which apparently obtain under present-day industrial conditions.

(3) INHALATION OF FELSPAR.

Felspar, like china clay, is an alumino-silicate and is also used in the manufacture of china and earthenware. Its empirical formula is $K_2O \cdot Al_2O_3 \cdot 6SiO_2$.

The lung changes produced by the inhalation of this dust at the standard concentration were slight up to one month after the last exposure, since they only comprised some proliferation and thickening of the alveolar epithelium. From two months onwards plaque-formation and areas of broncho-pneumonia are in evidence, although the amount of dust in the lungs undergoes some diminution. The last specimen examined (9 months after exposure) shows a mild fibrosis of the lung substance immediately surrounding the peribronchial and peri-vascular connective tissue. Dust appears in the bronchial glands one month after exposure, but its presence is not always constant.

The phagocytic response to this dust is slow and was not appreciable until one month after exposure. And, when it does occur, it is feeble.

The evidence, then, is that felspar, when inhaled under the conditions of these experiments, is a very harmful dust. Like china clay, it produces but little initial reaction, though severe and permanent lesions subsequently follow. Even after the short exposure to which the guinea-pigs were submitted felspar eventually produced a slight fibrosis as well as plaque-formation.

Felspar, though used in the china and earthenware industries, does not seem to be responsible for injury to the lungs of the workers, since, as pointed out to me by Dr White, it is used wet.

These experiments indicate that felspar, like china clay, must be regarded as a dust which would probably prove very harmful if inhaled by human beings.

(4) INHALATION OF GROUND PITCHER.

Ground pitcher is the term employed in the Potteries for crushed earthenware after firing. Dr White informs me that ground pitcher is not inhaled by workers in china manufactories. Further, its use is very limited, being restricted to filling up small holes, etc., in porcelain, for which purpose it is made up with water.

The inhalation by guinea-pigs of this dust produces marked initial lesions; the lungs of an animal killed 24 hours after the last exposure, show great proliferation and thickening of the alveolar walls, and also bronchitis. Broncho-pneumonia and plaque-formation appear three months after exposure, fibrosis from the fourth month onwards. The latest specimen, examined nine months after exposure, showed no improvement, normal tissue being very scanty. Phagocytosis is more active than in the lungs of animals exposed to china clay or felspar. Dust is found in the bronchial glands from three months onwards, from which time it seems steadily to accumulate therein.

The histological evidence furnished by these experiments is that while ground pitcher produces marked initial damage to the lungs, phagocytosis of the dust is too slight, and its onset too slow, for the onset of permanent pulmonary lesions to be avoided. The fibrosis caused by this dust was the most definite in any of these experiments.

Ground pitcher is not, to my knowledge, inhaled by any type of worker, though these experiments indicate that its inhalation by human beings would probably provoke very serious lesions.

(5) INHALATION OF PURE AMORPHOUS SILICA.

This dust was administered so as to test the effect on the lungs of silica in the amorphous as opposed to the crystalline state. The dust was dehydrated and rendered insoluble by heating.

The first changes (a few hours after exposure) comprise proliferation of the alveolar epithelium, also capillary engorgement and some haemorrhage into the alveoli. The onset of plaque-formation is early. Bronchiolitis and a patchy broncho-pneumonia are in evidence from the fourth month onwards. The animals killed 4 and 5½ months after exposure show a slight fibrosis. A specimen examined 8 months after exposure presented similar lesions, except that there are no plaques; another, after 13 months, shows sub-pleural plaques but no fibrous tissue increase. The last guinea-pig of this series, examined 23 months after exposure, shows marked bronchiolitis, plaque-formation, and several large areas of broncho-pneumonia, but no fibrosis.

Fibrosis was not constant in all the animals, presumably for the reason already suggested—viz. that the concentration of the precipitated silica was a "borderland" concentration.

The presence of plaques in the two latest specimens (13 and 23 months) shows that permanent damage was caused by this dust.

The above results, together with those of Mavrogordato (1918) on the inhalation of soluble silica, may be discussed here. Gye and Kettle (1922, 1 and 2) have shown that the subcutaneous injection of either soluble or insoluble silica promotes a typical lesion. The only difference observed after injection of the soluble, as opposed to the insoluble, form of silica was the more rapid onset of the lesion with the former. And this, according to Gye and Kettle, is due to the colloidal (*i.e.* soluble) silica acting directly as a tissue-poison, whereas the insoluble SiO_2 can only inflict damage after undergoing hydration—and thereby becoming soluble—in the tissues. Certainly, that would seem the logical conclusion to be derived from the experiments of these authors.

The effects, however, of the inhalation of silica and of siliceous compounds are difficult to reconcile with this view for the following reasons:

(i) Shale contains from 55 per cent. to 60 per cent. of silica (Geikie, *Text-book of Geology*, 4th ed.) of which 35.2 per cent. consists of quartz (Miller). Yet both human and experimental evidence is unanimously in agreement that shale can be inhaled in large quantities without harm.

(ii) The effect of soluble silica on the guinea-pig lung has been investigated by Mavrogordato. This author noted that the lungs of such animals, after 2 hours' exposure for 12 days, were "practically dust-free." In other respects they were normal.

(iii) On the other hand, the inhalation of pure precipitated (amorphous) silica, rendered insoluble by heating to *circa* 800°C ., caused pronounced and early lesions frequently accompanied by fibrosis.

One can only assume from (ii) and (iii) that the lungs are far more sensitive to insoluble than to soluble silica, which latter, according to Mavrogordato, causes no lesions whatsoever, presumably because it does not remain in the lungs.

I think that the above remarks show the necessity for extending the experiments of Gye and Kettle to the lungs. The injection of silica into subcutaneous connective tissue is one thing, the inhalation, or insufflation, of the same substance into the lungs, another.

In view of the fact that the lung is habitually dealing with dust throughout life, and that connective tissue is not, it seems premature to apply to lung the deductions from the response of connective tissue towards silica until that of the lung has been studied along similar lines.

There is another interesting point in connection with this dust. It is often maintained (see Parkes and Kenwood, 1920) that the degree of harmfulness of a dust is proportionate to the degree of angularity and of sharpness of its constituent particles. Haldane (1918) has already thrown doubt on this view. Now, precipitated silica and flint are chemically identical, but the former is amorphous, the latter crystalline. Yet the inhalation of the amorphous silica produced lesions even more severe than did the crystalline silica. Again, china clay is amorphous, yet very harmful to the lungs. The evidence of these experiments is totally opposed to the text-book view that the more finely crystalline a dust, the more damage will it inflict upon the lungs.

(6) INHALATION OF FLINT.

The inhalation of flint dust is notoriously deadly as is testified by the mortality in certain trades—especially flint-knapping—from pulmonary disease.

Flint dust is inhaled by certain types of workers in the china-making industry. China articles, before being baked are “placed”—*i.e.* carefully packed—in finely powdered flint in large fireclay pans called “saggars.” Some dust, in spite of all precautions, is generated during the placing of the china and its unplacing after it has been baked.

Flint used to be inhaled during the process of “china scouring” (*vide* Parkes and Kenwood, 1920). This comprised the removal of the flint dust from the fired china by brushing. The Managing Director of the Royal Worcester Porcelain Works informs me that mechanical scouring is nowadays the standard practice.

This series of experiments was carried out not so much to confirm what has long been known concerning the lesions caused by breathing flint, as to have a standard of comparison between this dust and the mixed flint and coal, and the precipitated silica series.

The early stages (27 hours after exposure) comprise small areas of haemorrhage and some proliferation of the alveolar epithelium. A patchy broncho-pneumonia and incipient plaque-formation was established one month later. Specimens examined 4, 7 and 9 months after exposure merely show an extension of these lesions. The last two animals of this series, killed 12½ and 13 months after dusting, show similar changes, much dust, but no plaques. Fibrosis was never present. The absence of plaques from the lungs of the last two specimens is curious; possibly the lungs originally contained small plaques which disappeared later in the manner suggested on p. 446.

The absence of fibrosis is in contrast with human experience of flint dust. It should be remembered that exposure over a long period is apparently necessary in man before fibroid changes are induced in the lungs, whereas the total number of hours of exposure to which the guinea-pigs were submitted was 24. Furthermore, as already mentioned, the scanty development of fibrous tissue in the guinea-pig lung is undoubtedly responsible for the difficulty in eliciting a fibrous tissue increase in response to dust inhalation.

(7) INHALATION OF PURE COAL.

Coal dust is generally admitted to be the least harmful of the many dusts inhaled by human beings; indeed, as already pointed out, there is both statistical and experimental evidence that in some ways it is actually beneficial. It is even possible that carefully graded inhalations of coal dust might be of use in the treatment of pulmonary tuberculosis.

Animals were exposed to coal dust so as to have a standard of comparison with the other dusts used in these experiments—notably the mixed flint and coal.

Pure coal, as is well known, elicits a rapid and marked phagocytic reaction. The lungs of a specimen killed six hours after exposure show general proliferation of the alveolar epi-

thelium and some broncho-pneumonia. Much dust is present. These lesions persist until the fourth month after exposure. Both dust and lesions have undergone diminution in two specimens examined nine months after exposure, while the lungs of the last animal, examined after 19 months, show a marked decrease in the amount of dust, much nearly normal lung substance and some patches of broncho-pneumonia.

Phagocytosis was active in all the specimens. Neither plaque-formation nor fibrosis were ever noted. Coal dust undergoes a rapid elimination from the alveoli, and the brisk phagocytic response of the lungs towards coal is the explanation for their tolerance towards repeated doses of this dust.

(8) INHALATION OF SHALE.

Shale is extensively used for dusting in coal-mines as a preventive measure against explosions. It is inhaled in considerable quantity by the miner. These experiments have furnished results confirming the previous observations of Beattie (1912) and Mavrogordato (1918).

The reaction of the lungs towards shale was found to be rather like their reaction towards coal, but less intense. The onset of phagocytosis is slower, though in the later specimens the dust has undergone considerable diminution. The lesions undergo regression from three months onwards after exposure. The last two specimens, killed 8 and 8½ months after dusting, show only slight thickening of the alveolar epithelium, and, in one of the animals, slight plaque-formation and a few small areas of broncho-pneumonia. Fibrosis was never noted.

Shale, then, is a dust which tends to be eliminated from the lungs, though not with the rapidity of coal. Its inhalation by guinea-pigs produced no permanent lesions except a very mild degree of plaque-formation in one animal. The coal-miner inhales shale dust mixed with coal and the latter is a notable stimulator of phagocytosis within the lungs. There consequently is no reason to deny the claim that shale dust is comparatively harmless—in spite of the fact that it contains over 50 per cent. of silica by weight.

(9) INHALATION OF IGNITED SHALE.

The shale used in these experiments, after heating to redness in the electric furnace, was administered exactly as for the preceding group.

It was thought that the reason why coal and shale are comparatively harmless when inhaled might be due to the presence of organic matter which stimulated phagocytosis in the case of these dusts.

The response elicited by this dust is similar to that provoked by ordinary shale. Phagocytosis is slow but steady. In the last specimen examined (11 months after exposure) the dust was considerably reduced in amount; there were a few small areas of plaque-formation, but no fibrosis.

Thus, the supposition which led to this experiment being made was not confirmed in practice. The organic matter in shale does not seem to be the factor inducing phagocytosis of this dust.

(10) INHALATION OF DRIED EARTH.

The aim of testing the effect of earth on the lungs was twofold: firstly, to ascertain if any lesions were produced by the inhalation of such a ubiquitous

substance, and, secondly, to compare the effects of dried with ignited earth as for shale.

The initial lesions are a thickening and proliferation of the alveolar epithelium accompanied by a patchy broncho-pneumonia. This persisted for as long as 9 months (when the last two animals were examined). Phagocytosis of the dust is but slight. Yet neither plaque-formation nor fibrosis have occurred. The lungs are microscopically negative for organisms.

While the phagocytic reaction towards this dust is slight, permanent and serious lesions have not been produced. The lungs show a peculiar tolerance towards it, and ordinary earth appears to constitute an exception to the generally correct view that the harmful dusts are those which are not eliminated.

(11) INHALATION OF IGNITED EARTH.

This was the same earth, administered in the same way, as the preceding group, but heated to redness in the electric furnace before administration.

The aim of the experiment was to test the supposition that it was the organic matter content of certain dusts which was the stimulant towards phagocytosis. Although dried earth produced but little phagocytic response it was thought that the absence of its organic matter might transform it into an actively harmful substance.

Unfortunately, an epidemic of pneumonia destroyed most of the dusted animals in both this and other groups.

The initial lesion (24 hours afterwards) is a patchy proliferation of the alveolar epithelium. The only animal (3½ months after exposure) which survived the epidemic does not admit of accurate interpretation in that there was an old, organised pleurisy over the greater part of the lobes. This, presumably, was a relic of a previous pneumonia contracted during the epidemic. Discounting this, and the underlying fibrosis, derived from it, the only lesions which can indubitably be ascribed to the dust are proliferated alveolar epithelium and scanty plaques.

In view of the absence of further material all that can be said regarding ignited earth is that its earlier lesions do not differ from those produced by ordinary earth.

(12) THE BRONCHO-PNEUMONIC LESIONS.

The type of broncho-pneumonia present in the lungs of the guinea-pigs employed for these experiments merits mention. Unlike broncho-pneumonia as ordinarily found in human beings, the cellular increase is primarily due to a proliferation of the cells of the alveolar epithelium. It is not chiefly an exudate of leucocytes except in such areas where eosinophil infiltration of the lung substance is also present. Further, this eosinophil invasion is secondary to the proliferation of the tissue cells. Only very rarely were necrotic foci seen. On the evidence furnished by human pathology many of these lesions could undergo modification (in time) in the sense that such damaged areas of lung could become functional again. This, in fact, has undoubtedly occurred in some of the animals which were kept for a sufficiently long time after exposure.

7. THE ORIGIN OF THE DUST-CELLS.

In addition to the other series of experiments, a special group of animals, exposed to a single but intensive exposure of three hours with coal, was examined. The aim of this was to avoid the inevitable overlap of the different stages of pulmonary reaction which occurs in animals subjected to repeated inhalations of dust.

As may be seen in the bibliographical section (see pp. 439 to 442) two schools of thought are in active controversy regarding the origin of the dust-cells. The one, animated by Metchnikoff (1901) and his pupils (1889), asserts that dust-cells are always leucocytes which have migrated from the blood stream. The other, of which Arnold (1885) may be regarded as the prototype, claims that dust-cells are always derived from the alveolar epithelium. Many recent observers hold the former view.

The evidence furnished by the examination of sections of dust-containing lungs is, on the whole, in favour of the epithelial origin of many of the dust-cells.

I have been led to this conclusion by the following observations:

1. The more actively a dust undergoes phagocytosis within the lungs, the more rapidly does proliferation of the alveolar epithelium set in. The reverse is also true; dust particles which evoke a poor phagocytic response can be seen plastered against the alveolar epithelium, which remains, sometimes for several weeks, relatively unchanged. This proliferation of the alveolar epithelium and the concomitant appearance of dust-cells suggests the derivation of the latter from the former.

2. Swollen cells, attached to the alveolar walls, and definitely spaced between normal alveolar cells, may often be seen projecting into the alveolar cavities. Such cells, which often contain dust particles, have every appearance of modified alveolar epithelial cells.

3. Only rarely have I observed anything which could be interpreted as the migration of leucocytes from the capillaries in the early stages of the ingestion of dust particles. Yet dust-cells, and proliferated alveolar epithelium, are usually much in evidence.

4. Metchnikoff (1901) claims that the dust-cells are invariably derived from leucocytes of the macrophage type, which have migrated very early in life from the blood stream into the alveolar walls. There they lie dormant, until awakened by dust particles or other foreign bodies. I can only say that I have never been able to find such cells in the alveolar walls of normal guinea-pig lung or even in dust-laden lungs soon after exposure.

5. Permar (1920, 1, 2, 3) holds that the dust-cells are derived from the endothelium of the pulmonary capillaries. Although some of the pigment may be taken up by such cells, I doubt whether the rôle played by the endothelial cells in these experiments was other than secondary for the following reasons:

- (a) Only very rarely have I noted anything which could be interpreted as

endothelial elements undergoing transformation into dust-cells as described and figured by Permar. This remark applies even to lungs in which active phagocytosis was taking place. On the other hand, stages in what I regard as the formation of dust-cells from the alveolar epithelium were clearly visible.

(b) I have often observed dust-containing cells forming an integral part of the alveolar epithelium. The position and connections of these elements are such that they can only be explained by regarding them as modified alveolar epithelial cells. If they are not, how have they come to form an integral part of the epithelium, and what has happened to the displaced alveolar cells?

6. The infiltration of the dust-laden lung is a general, but secondary, occurrence. Furthermore, such cells, in the guinea-pig lung, are usually eosinophils, or sometimes polymorphs, and never has it been possible to detect dust in these cells, in these experiments.

7. Sewell's (1918) experimental evidence—already summarised on p. 442—seems strongly to support the contention that dust-cells originate from the alveolar epithelium.

8. And, lastly, there is no *a priori* reason why modified alveolar epithelial cells should not engulf dust particles, since Briscoe's (1908) very careful investigations have shown that the first phagocytic reaction towards bacteria comes from the alveolar epithelium, and that only secondarily do the leucocytes of the blood stream intervene.

The above evidence, then, is in favour of the view that many of the dust-cells are mostly derived from the alveolar epithelium. I say "mostly" derived from this source for the following reasons:

(a) An unbiassed observer who looks through a sufficient number of sections of dust-laden lungs not infrequently encounters ambiguous appearances which can easily be interpreted so as to suit the requirements of any of the theories already discussed. I feel sure that this has only too frequently been done in the past.

(b) I can see no reason—except the tendency of mankind to pigeon-hole facts in narrow but convenient compartments—to regard dust-cells as *always* derived from leucocytes (or endothelial cells) or *always* from alveolar epithelium.

The plasticity of tissue-response is very great, but only too often plasticity on the part of the interpreters is lacking.

See also the Appendix of this paper (p. 468).

8. THE MODE OF ELIMINATION OF DUST PARTICLES FROM THE LUNGS.

These observations are based on the personal study of sections of guinea-pig lung. No startling differences were noted in the elimination of dust from the lungs of these and other animals—man included.

As is well known, the first stage in this process consists in the phagocytosis of the dust by cells which frequently appear to be derived from the alveolar epithelium.

Once dust particles have been engulfed they tend to undergo removal from the alveoli in one of the following ways:

1. *Bronchial elimination.* Some of the dust-cells become detached from the alveolar walls and lie free within the alveolar cavities. They ultimately pass up the bronchial tree and are eliminated from the lung. Doubtless the rôle played by the dust-cells, once they have absorbed the dust, is passive. The ciliary action of the bronchial epithelium, aided by the forced expirations of coughing, would be sufficient to eliminate both dust-cells and extra-cellular dust particles, provided these have attained a point above the terminal bronchioles—the epithelium of the latter being devoid of cilia.

2. *Lymphatic elimination.* The dust-cells remain attached to the alveolar walls for a considerable time; next, they enter the lymphatic vessels of the lungs.

The intra-cellular dust particles may pursue a slow course through the pulmonary lymphatics, finally coming to rest in the lymphoid tissue adjacent to the lungs. Thus, most of the intra-lymphatic dust comes to rest in the bronchial glands, next, in order of frequency and amount, in the tracheal glands, and, lastly, in certain of the abdominal viscera, notably the mesenteric lymph glands and the spleen. For dust to be deposited in either of these two latter sites it is necessary that it should have been inhaled over long periods, or in very high concentration. Dust within lymphoid tissue is largely, but by no means always, intra-cellular.

In the case of those dusts which do not actively stimulate phagocytosis the dust-cells may die within the pulmonary lymphatics. This gives rise, I think, to the small "intra-lymphatic plaques" already mentioned.

Dust may also be deposited in the peri-vascular lymphatics. Such dust is often extra-cellular. Haythorn, however, claims (1913) "that carbon pigment once taken up by the cells remains intra-cellular indefinitely unless freed by some process producing general necrosis of the tissues." I am inclined to doubt that this is always the case, having observed coal particles lying undoubtedly free in the cleft-like lymphatic channels of peri-vascular and peri-bronchial connective tissue. Yet the dust must (presumably) have been engulfed by dust-cells in order to pass from the alveolar cavities into the lymphatics. One can therefore only suppose that the dust-cells have disintegrated after taking up the pigment.

Haythorn's statement is open to yet another objection. It is now generally recognised that the life of blood leucocytes and other phagocytic cells is not commensurate with the life of the organism. The leucopoietic (*i.e.* white-cell forming) organs are in a state of constant activity during life. This implies that the genesis and death of the leucocytes occurs many times in the life of the organism as a whole. Yet Haythorn claims that a leucocyte—for he regards dust-cells as large mono-nuclear and transitional leucocytes—becomes endowed with a life span many times the normal, provided it has engulfed dust particles. This would seem to be highly unlikely.

3. *The dust remains in the lung substance.* In this case the dust is taken up by dust-cells which then degenerate and form the masses of large squame-like cells known as plaques. Plaques often form large conglomerations lying within the disorganised lung substance. Invasions by fibroblasts are common, and loss of vascular connections, almost invariable features in any fair-sized area of plaque-formation. Such a plaque, in contrast to the small "intra-lymphatic" plaques, is, I think, a permanent pulmonary lesion. There also often appears to be a difference in the distribution of these two types of plaque within the lung; the larger—and permanent—plaques may be found in any parts of the lung which are heavily laden with an inert dust. The "intra-lymphatic" plaques, on the other hand, are usually found in the lymphatics beneath the pleura.

The route taken by a dust which is undergoing elimination from the lung would appear to vary according to the nature of the dust.

Thus, the dusts which freely stimulate phagocytosis are eliminated both *via* the bronchi and *via* the lymphatic system. Such dusts comprise both coal and shale.

Those dusts which stimulate phagocytosis but scantily seem to be chiefly eliminated through the lymphatic system. Such dusts comprise china clay, felspar, ground pitcher, precipitated silica and flint. These are also the dusts which tend to remain in the lungs and cause serious, and sometimes permanent lesions.

In brief, the more harmful a dust is to the lungs the less far is it removed from the alveoli, and the less complete is its removal.

9. SUMMARY.

1. The lesions produced by the different dusts employed in these experiments, and the conclusions to be derived therefrom, have already been discussed in Section 6 of this paper (see p. 455, *et seq.*).

2. Plaque formation has been discussed. Evidence has been produced to show that the large plaques which tend to be formed in the parenchyma represent permanent pulmonary lesions. Small plaques, on the other hand, may be formed within the pulmonary lymphatics, in which case they may disintegrate in course of time. Such plaques have been termed "intra-lymphatic plaques."

3. Evidence has been brought forward against the view that the degree of harmfulness of a dust is proportionate to the degree of angularity and sharpness of its constituent particles. Nor has the supposition that the dusts which stimulated phagocytosis were those which contained organic matter been confirmed experimentally. Although silica may be regarded as a cell-poison and that this may account for the inability of the phagocytes to remove it, there are probably other factors than this, since shale—universally admitted to be one of the least harmful dusts—contains from 55 per cent. to 60 per cent. of silica. As to why one dust is harmful, and another not, is a problem as yet unsolved.

4. The origin of the dust-cells has been studied. Reasons have been given to justify the belief that dust-cells are frequently derived from the alveolar epithelium. This, however, does not exclude the possibility of some participation of leucocytes or endothelial cells in the phagocytosis of dust particles.

5. The chief modes of elimination of dust from the lungs have been discussed. It has been shown that the dusts which stimulate phagocytosis tend to be eliminated from the lung by both bronchi and lymphatics, while such elimination as occurs of the dusts which elicit but a feeble phagocytic response is through the lymphatics rather than the bronchi.

October 1923.

10. APPENDIX.

Since writing the section on the phagocytosis of dust particles (p. 464) I have had brought to my notice the following evidence bearing on this question.

Guieyessé-Pellissier (*C. R. Soc. Biol.* LXXXIII. 1920, p. 809, and *Ibid.* LXXXII. 1919, p. 1214) noted that intra-tracheal injections of olive oil in dogs and rabbits are rapidly absorbed and digested in the lungs. The cells responsible for this are derived from the alveolar epithelium. I have been able to verify this statement. All stages in the swelling up and desquamation of alveolar epithelial cells can be observed. Oil droplets can be identified in varying stages of digestion in both attached and free cells. The alveolar walls are largely formed of naked, beaded capillaries. Even after prolonged search I was unable to find more than two or three possible pictures of capillary endothelial cells undergoing detachment. In this case there can be no doubt that the part played by the alveolar epithelium in the phagocytosis of oil droplets is predominant, while the rôle of the endothelial cells is negligible.

Further, the statement of Permar and many others that "epithelia" are incapable of phagocytosis is extremely doubtful, since Guieyessé-Pellissier (*C. R. Soc. Biol.* LXX. 1911, p. 527) has described the phagocytosis of spermatozoa by the epithelial cells of the vas deferens subsequent to its ligation, while Regaud and Tournade (*C. R. Assoc. des Anat.* XIII. 1911 (Paris), p. 245) have noted a similar phenomenon in the epididymis following sterilisation of the testis by X-rays. In both cases a certain degree of de-differentiation of the epithelial elements occurred prior to their becoming phagocytic. I would also point out that the Sertoli cells of the seminiferous tubules normally ingest dead spermatozoa and other cellular débris.

On the other hand, Permar, after careful experimentation, states that the intra-alveolar phagocytes were endothelial in origin. Were Permar's experiments strictly comparable to the inhalation of air-borne particles? Possibly not, for the following reason. Permar first stained the endothelial leucocytes by intravenous injections of isamine blue, etc., then administered an intra-tracheal injection of carmine suspended in normal saline. Now, it is possible that the marked endothelial response, noted by Permar, was partly or largely

due to the introduction of a fluid into the lungs, an act which of itself must affect the local vascular system. At any rate, it is certain that the pulmonary response to dust particles in a fluid is not strictly comparable to the inhalation of dust particles in air.

One must admit, *either*, that Permar's observations were incorrect—a view to which I do not subscribe for a moment—or that, according to the nature of the dust particles and their medium of dispersion (*i.e.* air or saline) a different type of phagocytic response may be elicited, just as the different types of blood leucocytes respond to different stimuli.

It may also be pointed out that the absence of mitotic figures in adult alveolar epithelium is by no means evidence that cells cannot be produced from it. In addition to the possibility of amitosis, the study of the behaviour of epithelia both in tissue cultures and during regeneration has shown that migration of epithelial cells can occur on a large scale. I suspect that a phenomenon which I have noted *in vitro*, the detachment of alveolar epithelial cells followed by movement or growth of the adjacent elements to cover up the vacated space, occurs also in the dust-stimulated lung.

Finally, the detachment of alveolar epithelial cells from the alveolar walls is especially noteworthy in the cat's lung subsequent to inhalation of "Mustard Gas" in high concentration. In this case the alveolar epithelial elements form mulberry-like masses of large clear cells, surrounded by naked alveolar wall composed chiefly of elastic fibres and beaded capillaries (Carleton, *Report of the Chemical Warfare Committee*, No. 2, April, 1918).

May 1924.

EXPLANATION OF PLATES VI-X.

Low power microphotographs taken with a Zeiss 50 mm. planar and projection ocular. Medium and high power photographs taken with Zeiss apochromatic objectives and compensating oculars. All the microphotographs are untouched.

PLATE VI.

Fig. 1 ($\times 17$). *Control No. 1.* General view of normal guinea-pig lung. Note the scarcity of fibrous tissue.

Fig. 2 ($\times 90$). *Control No. 4.* Terminal bronchiole and adjacent alveoli of normal lung. Small branch of pulmonary artery above bronchiole. Note the flattened epithelial cells lining the alveolar cavities, and the absence of fibrous tissue except around the bronchiole and artery.

Fig. 3 ($\times 17$). *Control No. 2.* To right an area of thickened and proliferating alveolar epithelium—frequently present in the lungs of perfectly healthy guinea-pigs. Normal tissue on the left.

Fig. 4 ($\times 17$). *Control No. 6.* A small patch of chronic broncho-pneumonia; adjacent bronchi contain plugs of leucocytes and cell-débris. Normal tissue at the bottom. Small areas of broncho-pneumonia, surrounded by normal tissue, were noted in a few of the controls.

Fig. 5 ($\times 90$). *Inhalation of Flint and Coal (1st Series).* 24 days after last exposure. Section of unstained lung showing the abundant dust particles. Note the thickening of the alveolar epithelium in the dust-laden areas.

Fig. 6 ($\times 90$). *Inhalation of Flint and Coal.* 10 months after exposure. Area of broncho-pneumonia; adjacent bronchi contain leucocytes and dust-cells; LT=nodule of lymphoid tissue in lung—normal in the guinea-pig.

Fig. 7 ($\times 550$). *Inhalation of Flint and Coal*. 12½ months after exposure. High power view of dust particles within multinucleate dust-cells (*DC*). Alveoli nearly obliterated by the proliferation of the alveolar epithelium.

PLATE VII.

Fig. 8 ($\times 90$). *Inhalation of Flint and Coal*. 16 months after exposure. Chronic broncho-pneumonia over the entire field. *C* = "cuffing" of branches of the pulmonary vein by lymphocytes.

Fig. 9 ($\times 140$). *Same specimen as Fig. 8*. The higher magnification shows an area of plaque-formation (*PF*).

Fig. 10 ($\times 90$). *Inhalation of china clay*. 1 month after the last exposure. Lung nearly normal; some collapse at *X* (dark appearance of the microphotograph due to thickness of the section).

Fig. 11 ($\times 90$). *Inhalation of china clay*. 6 months after exposure. Area of broncho-pneumonia with massive eosinophil infiltration.

Fig. 12 ($\times 17$). *Inhalation of felspar*. 6 hours after the last exposure. Showing the early lesions. Most of the lung is normal; also area of thickened and proliferating alveolar epithelium.

Fig. 13 ($\times 90$). *Inhalation of felspar*. 12 months after exposure. Later stage of the lesions showing a large sub-pleural plaque, thickened alveolar epithelium and (above) an area of compensatory emphysema.

Fig. 14 ($\times 550$). *Inhalation of felspar*. 2 months after exposure. Showing an early stage of plaque-formation. *DC* = multinucleate dust-cells which have undergone concentration into plaques; *AE* = alveolar epithelial cells surrounding the plaques; *br* = a terminal bronchiole, its epithelium undergoing desquamation.

PLATE VIII.

Fig. 15 ($\times 35$). *Inhalation of ground pitcher*. 24 hours after the last exposure. On the left; area of broncho-pneumonia. On the right; more normal tissue.

Fig. 16 ($\times 550$). *Inhalation of ground pitcher*. 4 months after exposure. Incipient fibrosis in a dust-laden area. *FT* = strands of young fibrous tissue.

Fig. 17 ($\times 17$). *Inhalation of ground pitcher*. 6 months after exposure. Note the extensive chronic broncho-pneumonia.

Fig. 18 ($\times 400$). *Same specimen as Fig. 17*. Section of a bronchial gland. *M* = macrophages containing abundant dust particles.

Fig. 19 ($\times 17$). *Inhalation of ground pitcher*. 9 months after exposure. Showing a patch of broncho-pneumonia, small oval area of normal lung, and elsewhere thickened alveolar walls.

Fig. 20 ($\times 110$). *Inhalation of flint, felspar and china clay*. A few hours after the last exposure. Showing bronchitis and thickened and proliferating alveolar epithelium.

Fig. 21 ($\times 35$). *Same specimen as Fig. 20*. General view of the early lesions. A large area of dust-containing and proliferating alveolar epithelium.

Fig. 22 ($\times 152$). *Inhalation of flint, felspar and china clay*. 4 months after exposure. Haemorrhagic areas (darkly stained) into lung substance; great thickening of the alveolar walls and capillary engorgement are also present.

PLATE IX.

Fig. 23 ($\times 600$). *Same specimen as Fig. 22*. *FT* = fine strands of fibrous tissue in a dust-laden area.

Fig. 24 ($\times 79$). *Inhalation of flint, felspar and china clay*. 12 months after exposure. Illustrates the chronic lesions. *PF* = areas of plaque-formation; general thickening of the alveolar epithelium.

Fig. 25 ($\times 35$). *Inhalation of pure precipitated silica*. A few hours after the last exposure. Demonstration of the increased susceptibility of lobe as compared to apex. *Above*, portion of an apex showing a massive broncho-pneumonia; *below*, portion of a lobe of the same lung—some bronchitis and slight thickening of alveolar epithelium.

Fig. 26 ($\times 35$). *Inhalation of pure flint*. 22 hours after last exposure. Patchy proliferation and thickening of the alveolar epithelium; some bronchitis.

Fig. 27 ($\times 17$). *Inhalation of pure flint*. 12½ months after exposure. Section of apex; area of broncho-pneumonia near root of lung; elsewhere thickening of alveolar epithelium.

Fig. 28 ($\times 17$). *Inhalation of pure flint*. 13 months after exposure. Section of lobe. Bronchitis at bottom of field. A few small areas of slightly thickened alveolar epithelium. Note the greater severity of the changes in the apex (see Fig. 27) than in the lobe (as in this figure).

PLATE X.

Fig. 29 ($\times 17$). *Inhalation of coal*. Short exposure (two exposures of 2 hours each); animal killed immediately afterwards. Apart from some bronchitis the lung is normal.

Fig. 30 ($\times 17$). *Inhalation of coal*. 6 hours after exposure (usual period). Patchy broncho-pneumonia. Black masses of coal dust in the bronchi.

Fig. 31 ($\times 17$). *Inhalation of coal*. 19 months after exposure. More normal tissue than in the preceding photographs of the coal dust series. Broncho-pneumonia undergoing resolution. Still some bronchitis. Complete absence of plaque-formation.

Fig. 32 ($\times 17$). *Inhalation of shale*. 1 month after the last exposure. An area of broncho-pneumonia; to the left of this, normal lung.

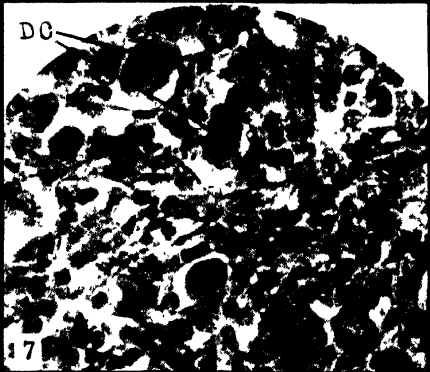
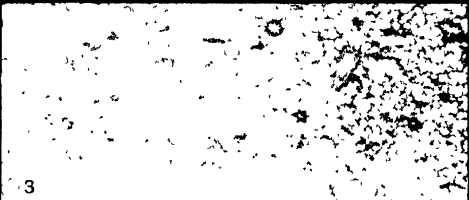
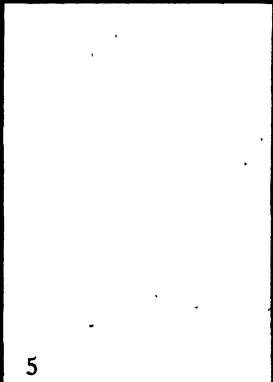
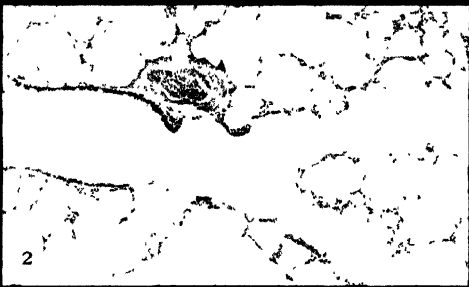
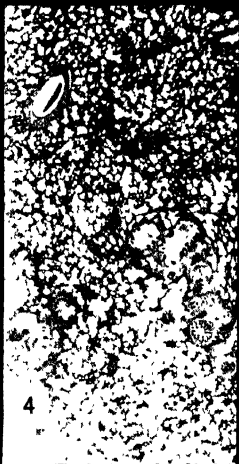
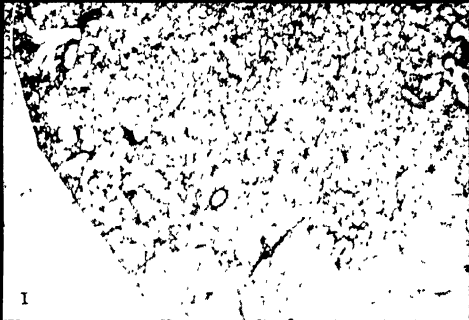
Fig. 33 ($\times 79$). *Inhalation of shale*. 3 months after exposure. An area of compensatory emphysema—a common feature in the lungs of dusted guinea-pigs.

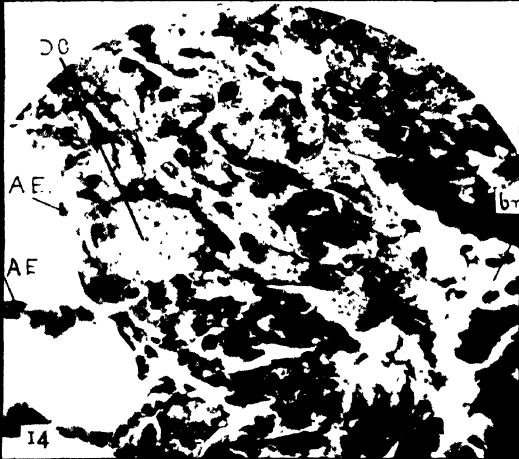
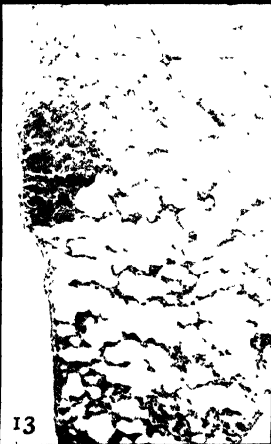
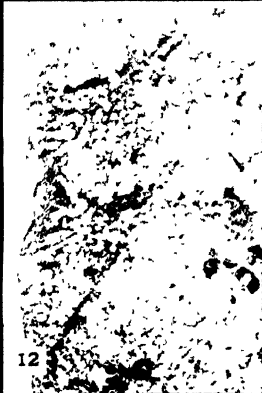
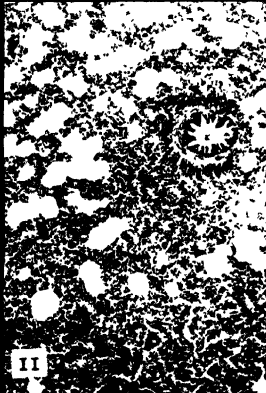
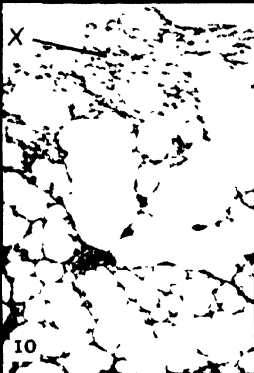
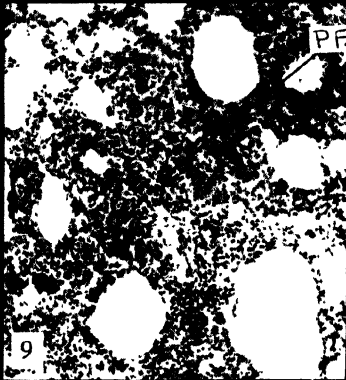
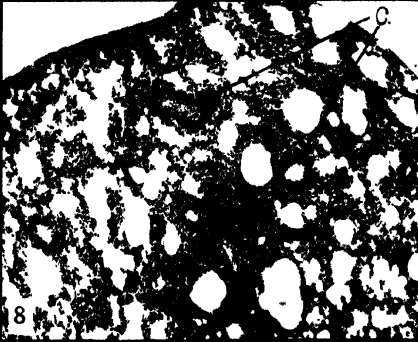
Fig. 34 ($\times 17$). *Inhalation of shale*. 8½ months after exposure. Mild bronchitis and, in places, slight thickening of the alveolar epithelium.

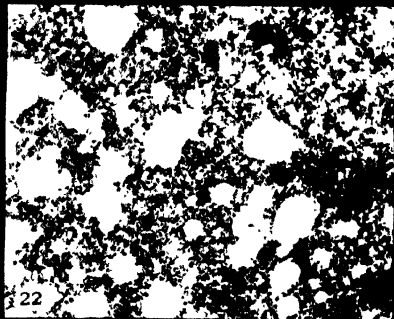
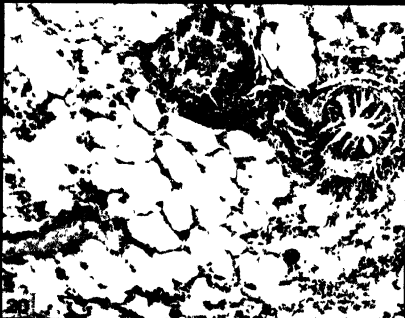
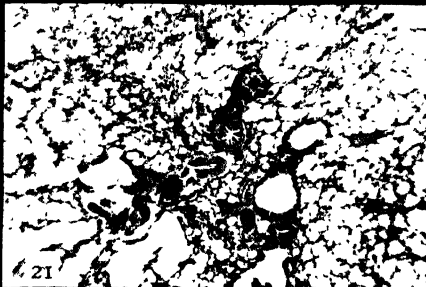
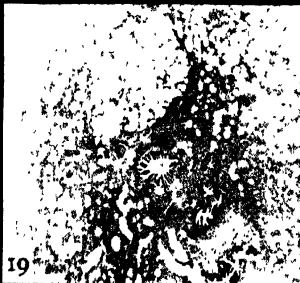
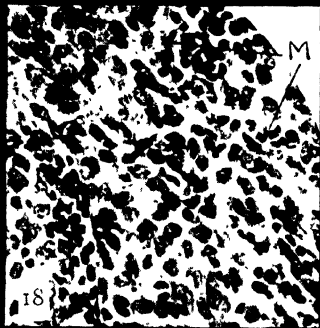
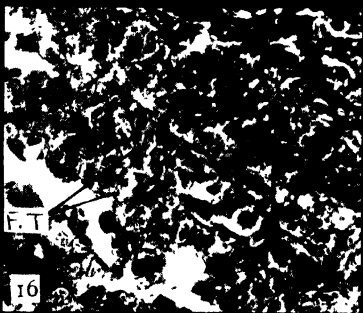
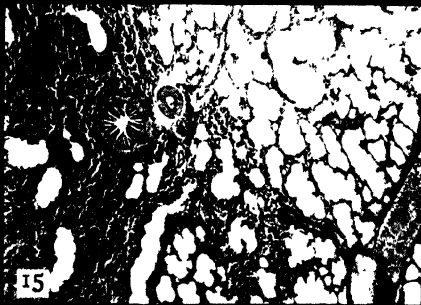
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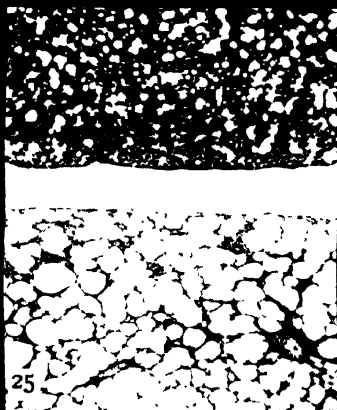
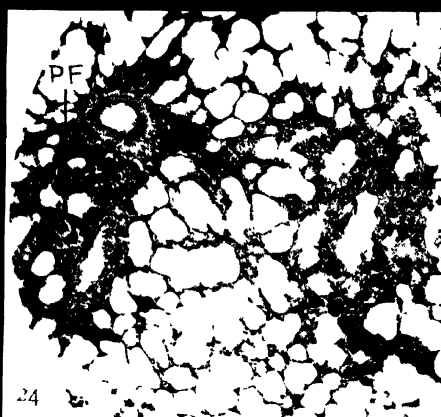
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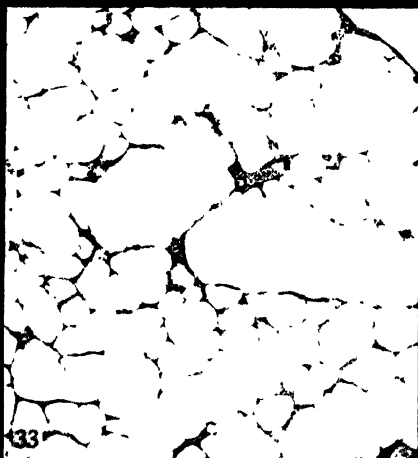
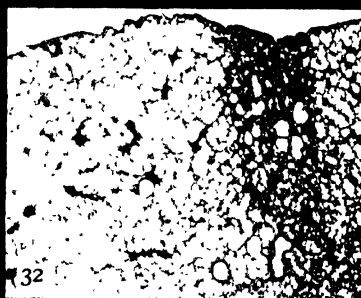
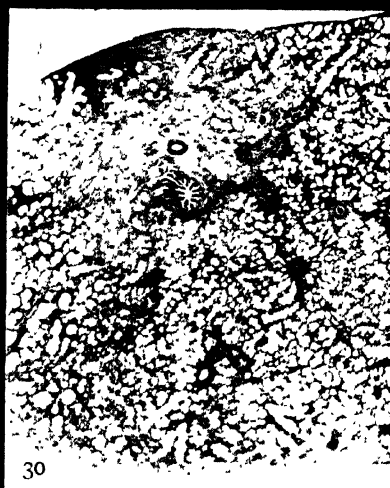
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THE STABILITY OF TETANUS TOXIN IN 50 % GLYCERINE AND OF TETANUS ANTITOXIN IN SATURATED SALT SOLUTION.

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THE Standard Tetanus toxin sent out by the Hygienic Laboratory of the United States Public Health Service is in the form of a dry powder, and the required quantities have to be weighed out.

This weighing must be done as quickly as possible, for the powder, being hygroscopic, is apt, on exposure, to absorb moisture from the air, in consequence of which the weighing is affected and the stock of standard toxin is liable to deteriorate in potency. This is a great inconvenience in the case of laboratories which are situated, as we are, where the air is usually moist. With the idea of simplifying matters a trial was made of a liquid toxin and as the results have so far proved satisfactory they are published as a possible matter of interest to other users of tetanus toxin.

Some of the Standard Tetanus toxin of the Hygienic Laboratory was, on January 17, 1921, dissolved in a mixture of equal parts of pure neutral glycerine and distilled water, and labelled G.T.T. 1. (Glycerinated Tetanus Toxin, No. 1.)

On January 21, 1921, a test was made to ascertain the M.L.D. of this mixture. Doses of 1/800 c.c., 1/900 c.c. or 1/1000 c.c. were injected subcutaneously in guinea-pigs weighing 340–380 grms. All three animals died on the 4th day.

On February 8, 1921, a further test was carried out:

Guinea-pig	Toxin dose	Result
350 grms.	1/1000 c.c. subcut.	+ 4th day
355 "	1/1200 "	Had tetanus but lived
	+ 4th day = death on 4th day.	

About one year later a similar test was performed:

25. i. 22.	Guinea-pig	Toxin dose	Result
	350 grms.	1/900 c.c. subcut.	+ 5th day
	340 "	1/1000 "	+ 6th "
	345 "	1/1100 "	+ 6th "

It is obvious that the M.L.D. had undergone little change during the twelve months that had elapsed since the previous test.

Tests having been carried out to ascertain the L + dose of this toxin it was taken to be 1/12th of a cubic centimetre. Between 19. iii. 21 and 21. vii. 22 this toxin was tested 33 times, one animal each time, in a dose of 1/12 c.c. against 1/220 c.c. of an antitetanus serum (Wilhelmina, 19. ix. 16).

The results of these tests were:

Day of death	3rd	4th	5th	
No. of animals	7	23	3	Total 33

The justifiable conclusion is that the glycerinated tetanus toxin remained stable during this period—the supply, unfortunately, did not last out longer—provided that the test serum also remained stable.

This test serum (Wilhelmina, 19. ix. 16) was an antitetanus serum which had been saturated with table salt, as it had been found that saturation with salt increases the stability of antitoxic sera.

Between 13. i. 21 and 11. i. 24, 1/220 c.c. of (Wilhelmina, 19. ix. 16) brined serum was tested 31 times, one animal each time, against 0.00075 grm. of the standard dry tetanus toxin issued by the Hygienic Laboratory, U.S. Public Health Service, with the following results:

Day of death	2nd	3rd	4th	5th	
No. of animals	1	10	17	3	Total 31

The question now arises, How does the brined serum compare with the standard tetanus antitoxin issued by the Hygienic Laboratory?

The answer to this question is given in the protocols of the comparative tests which follow.

1. xi. 20. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Tetanus Antitoxin.

Guinea-pig	340 grms.	0.00075 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 3rd day
	340 "	0.0008 "	+ end 2nd day
	340 "	0.009 "	+ 2nd day

L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined serum.

	345 grms.	0.00075 grm. std. toxin + 1/220 c.c. "Wilhelmina"	+ 4th day
	340 "	0.0008 "	+ 3rd day
	350 "	0.0009 "	+ end 2nd day

17. xii. 20. "Wilhelmina" brined serum against U.S.A. Standard Tetanus Toxin.

	350 grms.	1/220 c.c. "Wilhelmina" serum + 0.00075 grm. U.S.A. toxin	+ 3rd day
	340 "	" "	+ 3rd "
	365 "	" "	+ 3rd "

2. xi. 21. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Tetanus Antitoxin.

	340 grms.	0.0007 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 4th day
	340 "	0.00075 "	+ 4th "
	340 "	0.0008 "	+ 3rd "
	360 "	0.00085 "	+ 3rd "

L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined serum.

	345 grms.	0.0007 grm. std. toxin + 1/220 serum	+ 4th day
	340 "	0.00075 "	+ 4th "
	360 "	0.0008 "	+ 3rd "
	340 "	0.00085 "	+ 3rd "

19. iv. 22. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Antitoxin.

	340 grms.	0.00095 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 2nd day
	345 "	0.00085 "	+ 3rd "
	340 "	0.00075 "	+ 3rd "
	350 "	0.00065 "	+ 5th "
	340 "	0.00055 "	Tetanus—lived over 9 days

L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined serum.

	370 grms.	0.00095 grm. std. toxin + 1/220 c.c. serum	+ 2nd day
	350 "	0.00085 "	+ 3rd "
	370 "	0.00075 "	+ 3rd " Killed because of tetanus
	360 "	0.00065 "	+ 5th "
	375 "	0.00055 "	+ 7th "

L + G.T.T. 1 against U.S.A. Standard Tetanus Antitoxin.

375 grms.	1/10 c.c. G.T.T. 1 + 1/10 A.U. std. a.-t.	+ 3rd day
340 "	1/11 " " "	+ 4th "
350 "	1/12 " " "	+ 4th "
375 "	1/13 " " "	+ 5th "
375 "	1/14 " " "	Tetanus. Lived over 9 days

L + G.T.T. 1 against "Wilhelmina" brined serum.

365 grms.	1/10 c.c. G.T.T. 1 + 1/220 c.c. serum	+ 4th day
365 "	1/11 " " "	+ 4th "
360 "	1/12 " " "	+ 5th "
370 "	1/13 " " "	+ 7th "
370 "	1/14 " " "	Tetanus. Lived over 9 days

19. vi. 22. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Antitoxin.

340 grms.	0-0008 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 3rd day
345 "	0-00075 " " "	+ 3rd "
340 "	0-0007 " " "	+ 3-4 days

L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined serum

365 grms.	0-0008 grm. std. toxin + 1/220 c.c. serum	+ 3rd day
340 "	0-00075 " " "	+ 3-4 days
345 "	0-0007 " " "	+ 3-4 "

25. i. 23. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Antitoxin.

360 grms.	0-00085 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 3rd day
350 "	0-0008 " " "	+ 3rd "
340 "	0-00075 " " "	+ 3rd "
380 "	0-0007 " " "	+ 4th "
350 "	0-00065 " " "	+ 6th "

25. i. 23. L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined Serum.

355 grms.	0-00085 grm. std. toxin + 1/220 c.c. serum	+ 3rd day
345 "	0-0008 " " "	+ 3rd "
360 "	0-00075 " " "	+ 5th "
340 "	0-0007 " " "	+ 4th "
350 "	0-00065 " " "	+ 5th "

2 vii. 23. L + U.S.A. Standard Tetanus Toxin against U.S.A. Standard Antitoxin

375 grms.	0-00065 grm. std. toxin + 1/10 A.U. std. a.-t.	+ 5th day
380 "	0-0007 " " "	+ 4th "
380 "	0-00075 " " "	+ 4th "
365 "	0-0008 " " "	+ 3rd "
355 "	0-00085 " " "	+ 3rd "

L + U.S.A. Standard Tetanus Toxin against "Wilhelmina" brined serum.

340 grms.	0-00065 grm. std. toxin + 1/220 c.c. serum	+ 4th day
340 "	0-0007 " " "	+ 3-4 days
350 "	0-00075 " " "	+ 3rd day
380 "	0-0008 " " "	+ 3rd "
373 "	0-00085 " " "	+ 3rd "

For comparison with the previous results those with 0-00075 grm. of Standard Toxin against Standard Antitoxin and against brined Antitoxin may be grouped together.

	1/10 A.U. std. a.-t.	1/220 c.c. "Wilhelmina" brined serum
1. xi. 20	3rd day	4th day
2. xi. 21	4th "	4th "
19. iv. 22	3rd "	3rd "
19. vi. 22	3rd "	3½ days
25. i. 23	3rd "	5th day
2. viii. 23	4th "	3rd "

From these results we may conclude that:

(1) The antitoxic serum dissolved in a saturated solution of common salt remained sufficiently stable to justify its use as a test serum.

(2) That the glycerinated liquid tetanus toxin remained stable during the 18 months that the supply lasted; and

(3) That such a liquid tetanus toxin may be used for all preliminary testing.

An ordinary freshly prepared liquid tetanus toxin was mixed with an equal quantity of pure neutral glycerine and tested from time to time to ascertain its stability, 1/16th c.c. of the mixture being used with 1/220th c.c. of "Wilhelmina" brined serum. Between 27. ix. 22 and 18. vii. 23, 18 tests were carried out, one animal each time, with the result:

Day of death	3rd	4th	5th	6th	7th	
No. of animals	1	9	5	2	1	Total 18

Then the dose of toxin had to be increased to 1/14th c.c., and so it would seem that it would be safer to use a glycerinated solution of a dry powdered toxin precipitated by ammonium sulphate.

During the observations recorded above both toxin and serum were stored in the cold room.

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INDEX OF AUTHORS

	PAGE
ANDERSON, JAMES S., KINLOCH, J. PARLANE and SMITH, J. Epidemic Enteritis in Aberdeen due to Food Infections	89
BAMFORTH, J. A small Outbreak of Dysentery associated with an Unusual <i>Bacillus</i>	343
CARLETON, H. M. The Pulmonary Lesions produced by the Inhalation of Dust in Guinea-Pigs. A Report to the Medical Research Council. (With 5 Plates)	438
COBBETT, LOUIS. Vegetable Decomposition in Ditch Water Simulating Sewage Contamination. (With 1 Plate)	389
DUDGEON, LEONARD S. Acute Infection of the Urinary Tract due to a Special Group of Haemolytic Bacilli. (With 2 Charts)	348
DUNCAN, J. T. A "New" Salmonella from a Case of Enteric Fever. (With 4 Figures)	402
EAGLETON, A. J. and BAXTER, EDITH M. The Serological Classification of <i>Bacillus diphtheriae</i>	107
EASTWOOD, ARTHUR. The Capillary Endothelium in Relation to Anti-bodies	355
FORBES, J. GRAHAM. The Atmosphere of the Underground Electric Railways of London. A Study of its Bacterial Content in 1920. (With 1 Text-figure and 6 Charts)	123
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part II. (With 10 Charts)	12
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part III. (With 4 Charts).	37
GLENNY, A. T. and HOPKINS, BARBARA E. Duration of Passive Immunity. Part IV. (With 5 Charts).	208
GREENWOOD, MAJOR. The Life and Scientific Work of Arthur William Bacot. With a chapter by J. A. ARKWRIGHT. (With Portrait, Plate II)	265
KENT, S. S. SHRI. The Nutrition of Bacteria, with Special Reference to <i>Bacillus influenzae</i> (Pfeiffer). (With 1 Plate)	52
KHALED, Z. A Comparative Bacteriological Study of Bovine Abortion and Undulant Fever	335
LEARMONTH, J. R. On the Inheritance of Acquired Antibodies	100
MACCONKEY, A. T. On the Concentration of Serum by means of Sodium Sulphate	413
MACCONKEY, A. T. The Stability of Tetanus Toxin in 50 % Glycerine and of Tetanus Antitoxin in Saturated Salt Solution	473
MURRAY, E. G. D. Some Aspects of Meningococcal Virulence. A Report to the Medical Research Council of work carried out at the University of Cambridge Field Laboratories	175
PECKHAM, C. F. An Outbreak of Pork Pie Poisoning at Derby. (With a Foreword by William G. Savage)	69
PETRIE, G. F. A Commentary on Recent Plague Investigations in Transbaikalia and Southern Russia	397
RHODES, E. C. Notes on the Spread of Bacterial Infection. (With 2 Charts)	6
SADLER, WILFRID, KELLY, C. D. and MARTIN, G. R. On the Producing of Milk having a Low Bacterial Content	410
SAWYER, W. A., SWEET, W. C. and SHAW, A. ELAND. Institutional Hookworm Disease in a Non-Endemic Region. (With 1 Text-figure)	77
SHOUSH, A. T. Spontaneous Agglutination of the Cholera <i>Vibrio</i> in Relation to Variability	156
SIMPSON, J. V. A. A Report on the Ventilation of Schools. (With 1 Graph)	164

	PAGE
DE SMIDT, F. P. G. Notes on the Sporulation of <i>B. sporogenes</i> and other Anaerobes. A Report of the Food Investigation Board. (With 1 Chart)	314
DE SMIDT, F. P. G. An Apparatus for anaerobic Plate Cultivation in Hydrogen for separate Petri Capsules. (With 1 Plate)	325
SMITH, J. A Study of Diphtheria Bacilli, with Special Reference to their Serological Classification	1
TEH, WU LIEN- (TUCK, G. L.). A Further Note on Natural and Experimental Plague in Tarbagans	329
TOPLEY, W. W. C. and AYRTON, JOYCE. A Technique for Measuring the Excretion of Bacilli of the Enteric Group in the Faeces of Infected Mice. (With 1 Chart)	222
TOPLEY, W. W. C. and AYRTON, JOYCE. The Excretion of <i>B. enteritidis</i> (<i>aertrycke</i>) in the Faeces of Mice after Administration by Mouth. (With 10 Charts and 1 Text- figure)	234
TOPLEY, W. W. C. and AYRTON, JOYCE. The Segregation of Biological Factors in <i>B. enteritidis</i> (<i>Aertrycke</i>). A Report to the Medical Research Council. (With 1 Plate)	305
WATT, JAMES P. Typhoid Carriers in Aberdeenshire	417

INDEX OF SUBJECTS

	PAGE
Antibodies, inheritance of	100
Atmosphere, bacteria in	123
<i>B. enteritidis</i> in faeces	222, 234
<i>B. influenzae</i> , nutrition of	52
Bacteria, nutrition of	52
Bacterial infections, spread of	6
Carriers, typhoid	417
Cholera vibrio, agglutination of	156
Diphtheria bacillus, serological classification	1, 107
Ditch water, vegetable decomposition in	389
Dust, pulmonary lesions due to	438
Enteric fever due to new bacillus	402
Epidemic enteritis	89
Faeces, enteric group in	222, 234
Food poisoning	69, 89
Hookworm disease	77
Immunity (passive), duration of	12, 208
<i>Meningococcus</i> , virulence of	175
Plague investigations	397
Salmonella, a new	402
Serum, concentration of	413
Tetanus toxin, stability of	473
Typhoid carriers	417
Vegetable decomposition in ditch water	389
Ventilation of schools	164
Virulence of <i>Meningococcus</i>	175

I. A. R. I. 75

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1912	1912	1912
1913	1913	1913
1914	1914	1914
1915	1915	1915
1916	1916	1916
1917	1917	1917
1918	1918	1918
1919	1919	1919
1920	1920	1920
1921	1921	1921
1922	1922	1922
1923	1923	1923
1924	1924	1924
1925	1925	1925
1926	1926	1926
1927	1927	1927
1928	1928	1928
1929	1929	1929
1930	1930	1930
1931	1931	1931
1932	1932	1932
1933	1933	1933
1934	1934	1934
1935	1935	1935
1936	1936	1936
1937	1937	1937
1938	1938	1938
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2004	2004	2004
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2006	2006	2006
2007	2007	2007
2008	2008	2008
2009	2009	2009
2010	2010	2010
2011	2011	2011
2012	2012	2012
2013	2013	2013
2014	2014	2014
2015	2015	2015
2016	2016	2016
2017	2017	2017
2018	2018	2018
2019	2019	2019
2020	2020	2020



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